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## Description

### BACKGROUND OF THE INVENTION

#### Field of the Invention

This invention is related to testing devices having internal chambers into which fluids are drawn by capillary action, to methods of using such devices, and to methods of manufacturing such devices.

#### Background of the Invention

In the development of the diagnostics field, there has been explosive growth in the number of substances to be determined. For the most part, the medical field has looked to clinical laboratories for these determinations. The clinical laboratories have been dependent upon expensive sophisticated equipment and highly trained technical help to fulfill the manifold needs of the medical community. However, in a highly automated clinical laboratory, there is substantial need to perform one or a few assays on a stat basis with minimum equipment.

There is also an expanding need for having analytical capabilities in doctors' offices and in the home. There is a continuing need to monitor the level of drug administered to people with chronic illnesses, such as diabetics, asthmatics, epileptics, and cardiac patients, as it appears in a physiological fluid, such as blood. Tests of interest include prothrombin time, potassium ion, and cholesterol. Determining red-blood-cell count is also a common test. In the case of diabetic patients, it is necessary to determine sugar level in urine or blood.

Numerous approaches have been developed toward this end, depending to varying degrees on instrumental or visual observation of the result. Typical of these are the so called "dip-stick" methods. These methods generally employ a plastic strip with a reagent-containing matrix layered thereon. Sample is applied to the strip and the presence or absence of a analyte is indicated by a color-forming reaction. While such devices have proven useful for the qualitative determination of the presence of analytes in urine and can even be used for rough quantitative analysis, they are not particularly useful with whole blood because of the interferring effects of red blood cells, nor are they useful for making fine quantitative distinctions. Accordingly, there remains a need for the development of methods and devices capable of analyzing whole blood and other complex samples rapidly with a minimum of user manipulations.

Many small devices in the analytical area depend on the use of plastics having specified characteristics, such as optical transparency and machinability. Machinability refers here to the ability to produce chambers, channels, and openings of prescribed dimensions within the plastic device. Although numerous plastic devices have been devised, the fabrication techniques are not interchangeable because of differences in the devices or the desired measuring result. This is particularly true for devices containing channels or other chambers of small dimensions internally in the plastic material. The fine channels are difficult to produce entirely within a plastic matrix and, if prepared in the surface of two matrices to be sealed to each other, are readily deformed during many sealing processes.

Accordingly, there remains a need for new devices for use in methods of rapid analytical testing and for new methods of producing these devices.

#### Brief Description of the Relevant Literature

Powers et al., IEEE Trans. on Biomedical Engr. (1983) BME-30-228, describes detecting a speckle pattern for determining platelet aggregation, as does Reynolds, Light Scattering Detection of Thromboemboli, Trans. 11th Annual Mtg. of the Soc. for Biomaterials, San Diego, CA, April 25-28, 1985. Reynolds and Simon, Transfusion (1980) 20:669-677, describes size distribution measurements of microaggregates in stored blood. Of interest in the same area are U.S. Patent Nos. 2,616,796; 3,810,010; 3,915,652; 4,040,742; 4,091,802; and 4,142,796. U.S. Patent No. 4,519,239 describes an apparatus for determining flow shear stress of suspensions in blood. Ab Leo sells the HemoCue™ device for measuring hemoglobin. Also, see U.S. Patent No. 4,088,448, which describes a cuvette for sampling with a cavity which is defined in such a manner as to draw into the cavity a sample in an amount which is exactly determined in relation to the volume of the cavity by capillary force. However, the device of US 4,088,448 is not intended to measure flow, and therefore does not contain a reagent that is a member of a system capable of providing a detectible change in the flow rate of a liquid sample added to the device. The prior device is filled with a finite volume of a sample which is added to a reagent. By contrast, the invention uses the presence of a

reagent that modifies a characteristic of the sample associated with the flow in a capillary pathway.

Numerous plastic assembly techniques, particularly ultrasonics plastic assembly, is described in a book of the same name published by Branson Sonic Power Company, Danbury, Connecticut, 1979. Gallagan, Plastics Engineering, August 1985, 35-37 also describes ultrasonic welding of plastics. U.S. Patent 3,376,208 describes corona discharge, although for a different purpose. U.S. Patent 3,376,208 describes the use of an electric discharge to modify a film surface. A device used to transport liquids by capillary flow is described in U.S. Patent 4, 233,029. EP 0 104 881 discloses a device for testing blood transfusion compatability. A sample is drawn up into a porous plastic member by capillary action and a reagent which permits agglutination is then also wicked into the porous plastic member to cause a reaction. In contrast, in the present invention, the reagent is already present in the test device.

## SUMMARY OF THE INVENTION

The present invention provides fabrication techniques, the resulting devices, and techniques related to the use of such devices in which a defined chamber or channel is prepared within the internal space of a solid device. The devices typically call for the use of capillary force to draw a sample into the internal chambers of a plastic device. Such capillary flow devices, particularly capillary flow devices designed for a constant flow rate, typically include at least one capillary acting as a pump, usually for controlling the volume of the sample and the time period for reaction, a chamber, an inlet port, a vent, and a reagent in proximity to at least one surface of the device. The capillary and chamber provide for capillary flow due to surface action and for mixing of the assay medium with the reagent. The reagent is part of a detection system, whereby a detectable result occurs in relation to the presence of an analyte. The device and the corresponding method can be used with a wide variety of fluids, particularly physiological fluids, for detection of drugs, pathogens, materials endogenous to a host, or the like. In most cases an optical measurement is being made, which requires the selection of a transparent material. Devices of unusually advantageous properties can be prepared by injection molding acrylonitrile-butadiene-styrene copolymer (ABS) so as to form a depression of defined dimensions in the surface of at least one face of the polymer, increasing wettability of the surface in at least those portions defined by the depression using either plasma etching or corona discharge, providing energy directing ridges projecting from the surface of the plastic adjacent to the depression or in a second piece of plastic so shaped as to contact the area adjacent to the depressions in the plastic surface, and ultrasonically welding the two plastic surfaces so as to produce an internal chamber or channel of defined dimensions having an air-tight seal around the perimeter of the resulting chamber. Although the fabrication method can be used to produce internal chambers of any dimension, the method is particularly suitable for the production of chambers and channels of small dimensions that are suitable for inducing capillary flow.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are plan views of two embodiments of the subject invention, Fig. 1A employing a single capillary and chamber and Fig. 1B employing two capillaries separated by a chamber.

Figures 2A and 2B are a plan view and a side elevational view of a device employing three chambers.

Figure 3 is a plan view of a device for a plurality of simultaneous determinations.

Figure 4 is a plan view of an alternate embodiment, where the sample is divided into two separate channels.

Figure 5 is a plan view of an alternate embodiment employing an extended capillary path.

Figure 6 is a cross-sectional view of an embodiment showing the location of channels and energy directing ridges during the fabrication process.

Figure 7 is a block diagram of an electronic circuit suitable for use in an electronic capillary cartidge device to simulate the passage of blood through a capillary in a control cycle.

Figure 8 is a diagram of the physical location and electronic circuitry of a detector capable of determining depletion of sample in a reservoir of a capillary device.

## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

This invention provides devices and methods, where the devices rely upon capillaries, chambers, and orifices to pump fluids; to control measurement of fluids, reaction times, and mixing of reagents; and to determine a detectable signal. By varying the path through which the fluid flows, one can provide for a Variety of activities, such as mixing, incubating and detecting. The methods may involve the binding of

homologous members of a specific binding pair, resulting in complex formation. The complex formation can provide for a variety of events which can be detected by instrumentation or visual means. Alternatively, the methods may involve chemical reactions, for example, the detection of glucose or serum enzymes, which result in a detectable change in the sample medium. Since the devices rely upon capillaries and other chambers to control movement of fluids, accurate control of the dimensions of the internal chambers is essential. The fabrication techniques described later provide this accurate control.

The sample may be a fluid which is used directly as obtained from the source or may be pretreated in a variety of ways so as to modify its character. The sample will then be introduced into the device through an inlet port, which may introduce the sample into a chamber or a capillary. The sample will then transit the device passing through the capillary(ies) or chamber(s), where the sample will encounter one or more reagents, which reagents are involved in a system which produces a detectable signal. By having orifices which connect the pathway to the atmosphere at one or more sites, one can terminate the flow up to that site, so that the medium may be incubated for various times or movement stopped subject to the initiating movement, for example, Immediately prior to measurement.

Any liquid sample may be employed, where the sample will have a reasonable rate of flow due to the pumping of the capillary action. It is to be understood the capillary action is the sole driving force, relying on the surface action between the surface and the fluid. Where the sample is too viscous, it should be diluted to provide for a capillary pumping rate which allows for the desired manipulations, such as mixing, and for a reasonable flow time which will control the time period for the assay.

The sample may be derived from any source, such as a physiological fluid, e.g., blood, saliva, ocular lens fluid, cerebral spinal fluid, pus, sweat, exudate, urine, milk. The fluid may be subjected to prior treatment, such as preparing serum from blood, diluting pus, saliva; the methods of treatment may involve concentration, by filtration, distillation, dialysis, or the like; dilution, filtration, inactivation of natural components, concentration, chromatography, addition of reagents, chemical treatment.

Besides physiological fluids, other liquid samples may be employed where the component(s) of interest may be either liquids or solids and the solid(s) dissolved in a liquid medium. Samples of interest include process streams, water, soil, plants or other vegetation, air.

The analytes of interest are widely varied depending upon the purpose of the assay and the source. Analytes may include small organic molecules, such as drugs, hormones, steroids, neurotransmitters, growth factors, commercial chemicals, degradation products, drugs of abuse, metabolites, catabolites. Large organic molecules may be determined, such as nucleic acids, proteins, polysaccharides. Aggregations of molecules may also be of interest, particularly naturally-occurring aggregations such as viroids, viruses, cells, both prokaryotic and eukaryotic, including unicellular microorganisms, mammalian cells such as lymphocytes, epithelial cells, neoplastic cells.

Phenomena of interest which may be measured may be indicative of physiological or non-physiological processes, such as blood clotting, platelet aggregation, complement-mediated lysis, polymerization, agglutination.

The sample medium may be the naturally-occurring medium or the sample introduced into a liquid meidum which provides for the desired characteristics necessary for the capillary pumping action and the detectable signal. For the most part, aqueous media will be employed and to that extent aqueous media will be exemplary of the media employed in the subject invention. The aqueous media may be modified by the addition of a variety of miscible liquids, particularly oxygenated organic solvents, such as lower alkanols, dimethyl formamide, dimethyl sulfoxide, acetone. Usually, the solvents will be present in less than about 40 volume percent, more usually in less than about 20 volume percent. Besides other solvents, other liquid or solid additives may be included in the medium to modify the flow or other properties of the medium, such as sugars, polyols, polymers, detergents, surfactants, involved with changes in wetting, adherence, laminar flow, viscosity.

In addition to the components mentioned above, other additives may be included for specific purposes. Buffers may be desirable to maintain a particular pH. Enzyme inhibitors may be included. Other reagents of interest are antibodies, preservatives, stabilizers, activators, enzyme substrates and cofactors, oxidants, reductants, etc. In addition, filtration or trapping devices may be included in the device pathway, so as to remove particles above a certain size. The particles may include cells, viruses, latex particles, high molecular weight polymers, nucleic acids, by themselves or in combination with proteins, e.g., nucleosomes, magnetic particles, ligand or receptor containing particles.

The sample may provide the detectable component of the detection system or such component may be added. The component(s) will vary widely depending upon the nature of the detection system. One detection method will involve the use of particles, where particles provide for light scatter or a change in the rate of flow. The particles may be cells, polymeric particles which are immiscible with the liquid system,

latex particles, charcoal particles, metal particles, polysaccharides or protein particles, ceramic particles, nucleic acid particles, agglutinated particles. The choice of particle will depend upon the ease of detection, the dispersability or stability of the dispersion, inertness, participation in the change in flow, and the like. Particle sizes will generally be from about 0.1-100μ, more usually from about 5-15μ. Other phenomena which may be detected include changes in color, light absorption or transmission, fluorescence, change in physical phase.

The neat sample or formulated sample will be introduced into the entry port into the receiving unit of the device. The receiving unit may be a capillary or a chamber. The receiving unit may be used to measure the particular sample volume or may simply serve to receive the sample and direct the sample to the next unit of the device. The capillary units serve a variety of functions, including a measuring device for volume measurement, a metering pump for transferring liquid from one chamber to another, flow controller for controlling the rate of flow between chambers, mixer for mixing reagents, and detecting unit for detection. For the most part, the capillaries will serve as transfer units, flow control units and detection units. The chambers and capillaries may be used to define different events, e.g., areas of reaction, or different structural entities in certain embodiments of the method.

The capillaries will usually be of a substantially smaller cross-section or diameter in the direction transverse to the direction of flow than the chambers. The cross-section or length in the direction of flow may be similar or may differ by a factor of ten or more depending on the function of the capillary and the chamber. Capillaries will usually have diameters in the range of about 0.01mm to 2mm, usually about 0.1mm to 1mm. The length of the capillary, particularly the first capillary in the pathway, more particularly the first capillary when it is joined to the entry port, will be at least about 3mm, more usually at least about 5mm, and may be 1cm or more, usually not more than about 2cm, while subsequent capillaries may be shorter or longer, frequently at least one being longer, being as long as 10cm, usually not exceeding about 5cm.

The first capillary will initially control the rate of flow into the chamber which will usually serve as the reaction chamber. Thus, the capillary may aid in the control of the time with which the assay medium is in contact with reagent contained within or bound to the walls of the capillary and/or reaction chamber and the progress of the assay medium through the chamber. Other components which may affect the rate of flow in the chamber include baffles, walls or other impedimenta in the chamber, the geometry of the chamber, the reagent in the chamber and the nature of the surfaces in the capillary and chamber. Since in many instances the initial contacting of the assay medium and the reagent could affect the results, it is desirable that the contact be sufficiently slow that equilibrium can occur, as to dissolution, reaction.

The capillary control and use of relatively thin heat conductive walls allows for rapid heat transfer and isothermal conditions, or alternatively, thick walls can provide for adiabatic conditions. Thus, the small volume of fluid in the chambers and capillaries permits for rapid heat exchange or efficient thermal insulation. In addition, the thin capillaries permit optical measurements, particularly based on transmission of light, with optically dense samples, e.g., whole blood. There is the further opportunity for rapid efficient mixing, where by sonication the whole sample can be uniformly mixed.

The capillary provides the sole driving source for the movement of liquid through the device. Accordingly, careful fabrication of the capillary to exact dimensions is required. The device is normally employed in the horizontal position, so that gravity does not affect the flow rate. The composition of the walls of the capillary are selected so as to provide the desired degree of wetting and surface tension or the walls are modified to provide the desired physical properties. The device is employed without ancillary motive force, such as pumps, gravity.

The chambers also have a variety of functions, serving as protection for the reagent(s) mixing chambers for dissolution of reagent and/or reaction with reagent, volume measurement, incubation and detection, where the detectable signal is other than a signal associated with flow. The chambers will be primarily employed for mixing, incubating and for holding of liquids.

Depending upon the particular system, the length of the capillaries, their cross-sectional area, the volumes of the various chambers, and their length and shape, may be varied widely. One constraint on each of the capillaries is the necessity for their function providing capillary pumping action for flow. Therefore air leaks in the space surrounding the capillary (except for designed access ports) cannot be tolerated. In many instances, the chambers will also provide for capillary action while the flow rate which will be affected by the nature of the capillary surface will be primarily determined by the capillary action of the capillaries.

In order to minimize the handling of reagents by the user of the device, the reagents may be supplied within the device, where by mixing with the reagents occurs in the device. The reagents may be present either diffusively or non-diffusively bound to the surface of the device, that is, adhered, absorbed, adsorbed

or covalently-linked, so that the reagent may become dissolved in the fluid or may remain fixed to the surface. Where the reagents are diffusively bound (non-covalently and weakly bound), a variety of situations can be accommodated. One situation is where the liquid front dissolves all of the reagent, so that the liquid front receives a high concentration of the reagent and most of the reaction occurs at the liquid front. A second situation would be with an excess of a reagent of limited solubility. In this situation, the reagent may be present in the liquid medium at a substantially uniform concentration. A third situation is to have a deficiency of a reagent of limited solubility, so that only the early portion of the fluid will have a relatively constant reagent concentration. In many instances it is essential that the reagent be present in a defined area or reaction chamber, which makes fabrication of an internal chamber followed by later addition of reagent virtually impossible.

While for the most part, the reagent will be present in one or more units of the device, reagents also can be mechanically introduced by various techniques. For example, by employing a septum, a syringe may be employed for introducing a reagent. Alternatively, one could have an orifice and use an eyedropper or other means for introducing liquid reagent into the unit. Usually, unless essential, these alternative techniques will be avoided.

The reagent will vary depending upon the nature of the sample, the analyte, and the manner in which the detectable signal is generated. A chemical reaction will occur due either to the formation of covalent bonds, e.g., oxidation or reduction, hydrolysis, or non-covalent bonds, e.g., complex formation between ligand and receptor, including complex formation between nucleic acids.

The same or different reagent may be present in the various units, so that successive reactions can occur or a reagent continually supplied into the moving medium. Also, one could have a plurality of chambers and capillary channels. Frequently, the first unit will have a reactant. The chambers can be varied in size and purpose, providing the varying incubation times, varying reaction times, mixing of media from different capillaries. Any number of chambers may be employed, usually not exceeding six, more usually not exceeding about four, where the chambers may be in series or parallel. The size of the unit, either capillary or chamber, can be particularly important, where the reagent is fixed, so that the residence time in contact with the reagent will be affected by the area of the reagent contacted by the assay medium.

By employing various filtration or trapping devices (e.g., mechanical or magnetic), one can inhibit the transfer of particles from a capillary channel to a chamber or vice versa. In this way, red cells can be removed from blood, various components of the sample may be removed, or by employing divergent channels, one channel can have particles removed and the particles retained in the other channel where the two results may be of interest.

Arbitrarily, the use of the device will be divided into two different concepts. The first concept will involve a characteristic other than a change in flow rate. For the most part, this will involve the absorption, scatter or emission of light. A wide variety of protocols and reagents are available which provide for a change in the measured light, as a result of absorption, scatter or emission, in relation to the amount of analyte in the sample.

Labels which may be employed include enzymes, in combination with substrates, cofactors or inhibitors, fluorescers, combinations of fluorescers and quenchers, dyes. In some instances a chemical reaction occurs as a result of the presence of the analyte or with the analyte, which provides a detectable signal. By employing appropriate protocols, the amount of absorption or emission of light in the detection unit can be directly related to the amount of analyte in the sample.

Detection of a change in the rate of flow may be the signal which results from the reaction of the label or may be the result of a combination or a plurality of entities, which affect the rate of flow. The change in flow rate may be as a result of agglutination, polymerization, complex formation between high molecular weight compounds or aggregations.

The measurement of light, e.g., scatter, can be used to measure a change in the size population. This can be particularly useful for measurement of agglutination, clumping, clot formation or dissolution. A laser is able to distinguish particle size without a change in the flow rate. Small particles have a low frequency and a high amplitude; large particles (agglutinated particles) have a lower frequency (fewer total particles) and a higher amplitude (each particle is larger). Thus the change in particle size distribution may be detected by integrated noise employing known circuitry.

Various situations can be involved where the assay medium may have no particles (entities capable of scattering) or have particles, such as cells, latex beads, where the result of the reaction is to change the particle size distribution, including going from no particles to the formation of particles. There is also the opportunity to begin with particles, e.g., blood clots, and as a result of the reaction reduce the size and number of particles, e.g., dissolve the blood clots.

Protocols which may find use include those found in U.S. Patent Nos. 3,817,837; 3,839,153; 3,998,943; 3,935,074; 4,174,834; 4,233,402; 4,208,479; 4,235,869; 4,275,149; 4,287,300, whose relevant disclosure is incorporated herein by reference.

Because of the enormous diversity of protocols which are presently available which can be employed in the subject methods and devices, only a few will be illustrative and reference will be made to numerous patents which describe different protocols.

In a first exemplary protocol, a fluorescence measurement may be made, where one has a single capillary as the inlet, with the capillary coated with antibody to analyte. The sample is diluted with a buffered reagent containing a conjugate of analyte with fluorescer, whereby all of the fluorescent reagent will become bound in the capillary in the absence of any analyte in the sample. The capillary is then introduced into the sample and an amount of liquid withdrawn up to an indexed point on the capillary, whereby the capillary is then withdrawn from the sample and the liquid allowed to progress into the chamber. When the chamber is partially or completely full, the fluorescence off the chamber may then be read as indicative of the amount of analyte in the sample.

With enzymes, one could either vary the protocol or the device to prevent premature interaction between the enzyme and its substrate or inhibitor. Where a simple two-unit device is employed, employing a capillary and chamber, one could provide for using a combination of enzymes, referred to as channeling, where the product of one enzyme is the substrate of the other enzyme. In this manner, one could have in one unit a second enzyme combined with the substrate of the first enzyme, while the second unit having the first enzyme combined with the substrate of the second enzyme.

One could modulate the reaction by various means. For example, one could have antibody to analyte in the first unit and combine the sample with a buffered solution of antibody-first enzyme inhibitor conjugate. Thus, the amount of enzyme inhibitor which would enter the second unit would be related to the amount of analyte in the sample. Instead of having a two-unit system, one could have a three-unit system, where the first unit mixes the sample with the enzyme inhibitor conjugate avoiding the necessity to combine the sample with a liquid medium. Where the sample is colored, such as blood, it may be necessary to filter out or trap red blood cells; to allow for development of color or fluorescence or to find a wavelength range, where one can read the development of a particular light-absorbing material.

By employing a plurality of units, one can use a single enzyme, where the enzyme is conjugated to the analyte. By having enzyme-analyte conjugate in a first unit, followed by antibody to anlayte in a second unit and employing a third unit containing enzyme substrate as the reaction chamber, the measurement can be made in the third unit.

By employing combinations of filters and particles one could also achieve similar effects. For example, one can employ enzyme analyte conjugates in the first unit which completely dissolve in the assay medium. A second unit may then contain the particles containing antibody to the analyte. The amount of enzyme-analyte conjugate which binds to the antibodies will be dependent upon the amount of analyte in the sample. By having a filter at the exit of the second unit, all of the particles will be trapped at the filter, and only enzyme conjugate which is unbound will pass through the filter to the third unit. The third unit may then contain the enzyme substrate, so that reaction of the enzyme with the substrate can be monitored.

For the detection of change in flow rate, a wide variety of systems may be employed. Of particular interest is the natural system involving clotting, which is convenient when the sample is a blood sample. Thus, by adding one or more components of the clotting cascade or a component which activates the naturally-occuring components present, the clotting can provide for a change in the flow rate. Particularly, these reagents may include thromboplastin, factors I, II, IV, V, VII, VIII, IX, X, XI and XII. These components can be added individually or in combination. Particular combinations include factors of the intrinsic pathway (VIII, IX, XI and XII) or extrinsic pathway (III, VII) with the common pathway factors I, II, V, X, XIII. The clotting assay can be used to determine a wide variety of analytes of interest. The clotting assay may be used for the detection of the presence of anti-clotting agents or clotting agents. In addition, the clotting assay can be used for the detection of the level of a particular component required for clotting or a componenet involved in the dissolving of clots. Illustrative analytes include the various factors indicated above, warfarin, tissue plasminogen activator, heparin, streptokinase, Vitamin K, anti-platelet drugs, protamine sulfate.

The clotting assay can also be used to measure any analyte of interest by having the analyte associated with a factor necessary for clotting. For example, by conjugating thromboplastin to a compound capable of competing with the analyte for a receptor, where the thromboplastin-receptor complex is inactive, one could determine the presence of the analyte. The sample would be mixed with the receptor for the analyte so that the binding sites of the receptor would be occupied by any analyte present. The thromboplastin conjugated to the analyte or mimetic analog would be the reagent in the reaction chamber.

All the other components necessary for clotting would be included in the sample along with the antibodies for the analyte, where such other components were not naturally present in sufficient amount in the sample. The receptor sites which were not occupied by analyte would bind to the analyte conjugated to the thromboplastin where the resulting specific binding complex would lack thromboplastin activity. The remaining uncomplexed thromboplastin conjugate would be active and initiate clotting. By employing appropriate standards, one could relate the time to flow stoppage to the amount of analyte in the sample. Or, one could provide that flow stoppage did or did not occur with an amount of analyte above a threshold level.

Besides coagulation, agglutination, precipitation, or plug formation by other means, or, as appropriate, increasing viscosity can be used as part of the detection scheme.

For plug formation or slowing of flow by other than clotting, particles will usually be included in the medium which become cross-linked in relation to the amount of analyte present. This can be achived by employing receptors and ligands specific for the receptor so that non-covalent binding provides for multiple linkages between particles with a resulting cross-link structure which can serve as a plug. By using such particles as S. aureus, which specifically binds to immune complexes, red blood cells, synthetic or naturally-occurring particles to which ligands, rheumatoid factor, antibodies, naturally-occurring receptors, are conjugated, the cross-linking of the particles can be retarded or enhanced by the presence or absence of the analyte. Thus, in the case of antigens, the antigen may serve as a bridge between different antibodies, while in the case of haptens, individual haptens may inhibit the cross-linking resulting from a polyhaptenic molecule.

Various illustrations can be made of the different combinations which may be employed in the flow modulation methods of the subject invention, For example, the reagents could include an agarose bead to which is bound polyclonal antibodies to an antigen of interest or monoclonal antibodies where different particles are specific for different epitopic sites of the antigen. The sample need only include the detection component and any of the analyte which may be present. The sample would mix with the antibody-conjugated particles in the reaction unit and then flow into the exit unit. The presence of the analyte would result in cross-linking of the particles with large amorphous particles providing a greater drag on flow smaller particles. As the accretion of particles increased, ultimately a plug would form and flow would stop. In the case of haptens, the sample could be combined with antibodies to the haptens. The reagent could be hapten conjugated to Porglas beads. Available antibody would be capable of cross-linking the hapten-conjugated beads so as to ultimately provide a plug which would inhibit further flow.

Similar techniques could be used with hemagglutination where the particle is a red blood cell to which particular antigens are or have been bound. A sample which might contain cells having as part of their surface membrane the same antigen would be combined with the antigen conjugated red blood cell particles. The reagent would be antibody to the antigen. The rate of formation of the plug would vary depending upon whether cells containing the same antigen were present or absent.

A further illustration would involve polymers to which are attached polyclonal antibodies, where the polymers are selected so as to have only moderate solubility in the assay medium. Binding to antigenic bridges would result in desolubilization.

The polymers could be conjugated with nucleic acid sequences complementary to preselected sequences. The sequences would not be complementary to the nucleotide sequence of interest or to each other. The sample could be a lysate of a virus or pathogenic organism in an appropriate aqueous medium, where the genomic polynucleotides could be sheared. The sample would be combined with the polymeric reagents. The sample would then react in the reaction chamber with a nucleotide sequence which was a hybrid having a sequence complementary to the nucleic acid sequence of interest and a sequence complementary to the sequence bound to the polymer. Thus, the nucleic acid of interest would serve as a bridge to cross-link the polymeric molecules so as to set up a polymeric structure which would substantially slow the flow in the capillary and could, if desired, provide a plug.

Another exemplification would employ dual receptors, e.g., antibody to analyte and antibody to a particle, such as an antigen of a red blood cell (RBC). In quantitating a multivalent antigen, in the presence of antigen, cross linking would occur between the antigen, dual receptor and RBCs to form large complexes modulating the flow rate, while in the absence of the antigen no complexes would be formed. The assay could be carried out by combining the sample with RBCs before introducing the sample into the device or by providing RBCs in the sample receiving chamber. The reaction chamber would have the dual receptor where complex formation would be initiated.

For monovalent or haptenic analytes, the assay would be modified by employing a polyhapten reagent which could be added to the sample prior to introduction of the sample into the device. The presence of hapten would reduce complex formation in contrast to the result observed with the multivalent antigen

analyte.

Any system which results in a change in flow velocity or can be coupled to a reagent or system which affects flow velocity may be measured. Various systems have already been indicated which result in changes in flow rate. Other systems which could be coupled to compounds of interest are light initiated catalysis of polymerization, cellular aggregation initiated by lectin cross-linking, enzymatic reactions resulting in polymer initiation in conjunction with water-soluble monomers, e.g., hydroxyalkyl acrylates.

It is evident that the system permits a wide variety of variations which allows for a variety of protocols and reagents. Thus, any substance of interest which allows for flow in a capillary can be detected in accordance with the subject invention.

The flow in the capillary channel unit can be detected by various techniques which allow for detection of fluid flow, e.g., flow sensors or pressure sensors, or by having a detectable component in the assay medium, which can be detected visually or by diode assay. Techniques which allow for fluid flow determinations include the use of means for measuring triboelectricity, means for detecting the rate of passage of liquid, detecting Doppler effects, or the like. Preferably, a component is used in the medium which allows for flow detection by detecting the passage of the component through the first capillary channel exiting a receiving chamber.

Flow can be detected by the creation of a speckle pattern resulting from the movement of particles in the first capillary channel and the passage of a coherent light source, e.g., laser beam, or an LED, through the channel. (See, Powers et al., supra.)

A speckle pattern results from the interaction of particles and coherent light. Flow (motion) of the particles makes the speckels move with a frequency associated with the flow rate and the light or speckle fluctuations can be detected by a photodetector. The photodetector is designed to detect an area not greater than about the size of a speckle. A plurality of photodetector elements may be employed for detecting different areas and averaging the signals from each area. The speckle pattern can also be used to determine the size of the particles by analysis of the size of the speckles.

By employing a photodetector, the change in the light pattern as a result of a change in the rate of flow can be determined by appropriate electronic means, such as photodiodes or phototransistors, which would feed the electrical signal resulting from the fluctuating light to an appropriate circuit. Particularly easy to distinguish is a flowing liquid from a stationary liquid. Thus, the slowing or stoppage of flow can be readily detected and the change in rate of flow or the time of passage through the first capillary can be determined from the beginning of flow to the stoppage of flow.

One possible problem that can occur in capillary flow devices of the invention is depletion of blood or another sample from the reservoir prior to the stoppage of flow caused by the detectable event being measured, such as coagulation. When the liquid in the reservoir is drawn down so that essentially no more fluid is present in the reservoir, flow will stop since capillary forces will then be operating in both directions. Accordingly, it is useful to have a means of detecting this anomolous result in order to avoid a measurement of flow stoppage caused by this event being taken to represent the measurement flow stoppage.

Since the actual device containing the capillary channels and other chambers is typically a flat cartridge that is inserted into an instrument which makes the various electronic measurements, detection of reservoir depletion can be accomplished by embedding various sensors into the electronic device that holds the reaction cartridge.

Since the reservoir is generally external to the electronic device so that blood or another fluid can be applied directly to the reservoir, measurement of depletion of fluid in the reservoir typically takes place in the presence of ambient light and other ambient conditions, variation in which must be accounted for in any measurement technique. One suitable measurement technique is to apply modulated light to the reservoir in a region adjacent to the capillary leading to the reaction chambers and other parts of the apparatus. In fluids containing particles, such as red blood cells in blood, light is scattered in all directions through the fluid even though the light is applied perpendicular to the reservoir. Some light will be scattered down the entry capillary, which will then act as a light guide. However, the presence of particles in the fluid present in the capillary will again result in scattered light which passes out through the transparent walls of the light guide (capillary), where it can be measured by a photodetector. The capillary channel filled with blood can be considered to be a leaky waveguide for light, because a difference in refractive index between the blood (high refractive index) bounded by a low refractive index material (capillary channel) will provide light guidance, while the presence of red blood cells will scatter the light through the walls of the capillary channel, thereby providing the leaking effect. Since light will only be scattered in the presence of red blood cells or other particles, a detector located in close proximity to the channel will detect the scattered light. The modulation of the applied light will isolate the detector from ambient interferences. If the light is

modulated at a defined frequency and detection electronics are sensitive only to that frequency, ambient effects will be eliminated. The modulation applied to the light can be of any type, such as sinusoidal waves or chopping, as long as the modulation can be both created and detected by electronic or mechanical means. Interferences from ambient light can further be eliminated by using infrared light, which offers additional advantages (when blood is the sample) of enhanced scattering and transmission.

This technique for detecting depletion of fluid in the reservoir offers several advantages over other techniques. Detection of fluids in capillary channels is normally accomplished by measuring changes in absorption or transmission of light passed through the channel. However, in certain instances this will not be possible because of the physical restrictions on the reservoir and its location in the capillary device and the electronic apparatus into which the capillary device is inserted. For example, the size of a finger, if blood is being obtained from a finger stick, will require that the reservoir be separated sufficiently from the electronic device to allow the finger to be placed onto the reservoir. This will mean that both sides of the capillary device adjacent to the reservoir are not in contact with the electronic apparatus since at least one side must be accessible for the finger.

With the method discussed here, there is no need to have both sides of the capillary available for transmission and detection of light. Because a scattering effect is used, the detector can be present either on the same side of the capillary on which light is applied, on the opposite side, or in any other physical relation as long as the detector is located adjacent to the channel.

An additional useful control device is some means for simulating blood flow through a capillary channel in order to determine whether the electronic apparatus into which the cartridge is being inserted is fully operational. Numerous means of accomplishing this result are available, but one useful technique not believed to be previously used in any similar manner is described below.

As described previously, one useful technique for measuring blood flow is to detect the presence of the speckled pattern that results from the interaction of particles and coherent light. Any technique that simulates blood flow when such a detection system is being used will need to simulate the speckled pattern of light. Since the detector and the coherent light source are typically located in a close spatial relationship directly opposite each other so that insertion of the capillary device will result in light from the coherent light source passing directly through a channel in the device to the detector, simulation of blood flow requires insertion of some device into the electronic apparatus that can modulate the light beam. While this could be accomplished using a second device that could, for example, produce modulated light, a useful technique is to include electronics and modulating devices directly in the capillary device so that each capillary cartridge can be used to determine the operating characteristics of the electronic apparatus containing the coherent light source and detector immediately prior to actual measurement being taken. However, this requires that the speckled pattern generator be such that it will not then interfere with the actual measurement. One means of accomplishing these results is to include a liquid crystal display-type apparatus at the location where measurement is being made. The liquid crystal material is selected so as to rotate polarized light that passes through it, the typical means by which liquid crystals operate. Polarizer filters will be present, either in the cartridge itself or in the electronic apparatus into which the cartridge is inserted that will result in the passage of light through the polarizing filters when the liquid crystal device is turned off. However, when the liquid crystal device is activated by application of a voltage, light passage will be blocked.

Typically, when the liquid crystal device is activated, it rotates the polarization of the laser beam, thereby reducing the passage of a light and generating light amplitude fluctuations, which are detected as being equivalent to the moving speckled pattern generated by passing coherent light through the thin film of particle-containing fluid that would normally flow down the capillary channel.

A low viscosity liquid crystal material having a high refractive index change (thereby enabling rapid fluctuations) is desirable. A typical design uses a crystal oscillator and a chain of binary counters from which the liquid crystal display driver signals are derived as well as the time base for the measurements to be taken.

In order to further consideration of the invention, a number of illustrative devices which may be used will now be considered. As already indicated, the device will have at least one capillary channel unit, one chamber unit, an entry port, a vent, and a reagent bound to the surface.

The device will be fabricated from materials with the appropriate physical properties, such as optical transmission, thermal conductivity, and mechanical properties, and which allow for uniform coating and stability of reagent, as well as medium compatibility, for example, blood compatibility. Where blood is the medium, the material should be configured to assure good blood flow stoppage or slowing once clotting is initiated. For this purpose, suitable plastics include those for high surface free energies and low water sorption, including PETG, polyester (Mylar$^{R}$), polycarbonate (Lexan$^{R}$), polyvinyl chloride, polystyrene, and SAN. A particularly preferred plastic is acrylonitrile-butadiene-styrene (ABS), particularly ABS supplied by

Borg Warner under the tradename Cycolac. However, since these plastics are hydrophobic and exhibit poor reagent coating and poor blood flow, the plastics can be rendered hydrophilic by treatment with argon plasma, using a plasma etcher or corona discharge. Suitable conditions are 10-25 watts at 13.56MHz and 133,3 Pa (one torr) chamber pressure for 5-10 min. Alternatively, a protein, e.g., albumin coating, can be used in some instances by passing a solution through the device having from about 1-5% serum albumin, allowing the solution to stand for 30min., wiping and drying. Other modifications may also find application. Plasma etching and corona discharge provide markedly superior flow control characteristics and reproducibility.

The device can be fabricated in a variety of ways. The receiving and reaction chambers can be formed in the plastic sheed by vacuum forming (PETG), injection molding (PETG, polystyrene, SAN), or hot stamping. Capillaries may be formed by etching a channel into the plastic. The device can be sealed by placing a cover slip (with appropriate vent holes at the inlet and vent) on the base sheet, and sealing with ultrasonic welding or by solvent bonding. Of these techniques, markedly superior products are obtained by injection molding of the plastic device in pieces so as to form a depression in at least one surface of at least one plastic piece. ABS polymers are particularly suited to injection molding and additionally provide a clear plastic which is suitable for numerous optical detection techniques. ABS polymers are also suitable for ultrasonic welding. It is preferred to form the chambers from two substantially flat plastic pieces in which the capillaries and other chambers are formed by producing matching depressions in two surfaces of two different shaped plastic pieces. It is preferred that on one of the pieces ridges, known as energy directors, completely surround the depression in a closely spaced relation so as to form a surface of first contact when the two pieces are placed together. When ABS is used, the ridges are typically 0,1905±0.0127 mm (7.5 mil±0.5 mil) above the surface of the plastic. The ridges are typically formed in the shape of a triangle, typically an equilateral triangle. The center of the ridge is typically 0,4445±0,0127 mm (17.5±0.5 mils) from the edge of the depression that will form the chamber. Use of such energy directors with ultrasonic welding produces a highly reproducible seal around the edges of the internal chamber that is formed when the two sheets are ultrasonically welded together. Access ports are typically formed by molding or drilling holes into the depressed surfaces of the individual plastic pieces prior to welding. Accordingly, the welded ridges form a complete seal around the lateral edges of the internal chambers.

Alternatively, the pattern can be dye cut in a double-sided adhesive tape (e.g., 3M No. 666 tape, Fasson Fastape A) of appropriate thickness which is then sandwiched between a plastic base and cover slide. Or, the sandwiched layer may be die cut from a plastic piece of appropriate thickness which would be coated with adhesive and sandwiched in the same manner as the tape. The adhesive could also be silk-screened onto the base to give a raised pattern of desired thickness.

The sheet thickness of the device in the region of the capillary channels will generally be equal to or exceed about 0,0508 mm (2 mil) to prevent compression due to the capillary action. In the embodiment involving the sandwich, each of the plastic layers comprising the top and bottom will be at least about 0,254 mm (10 mil) thick.

While other materials may be used for fabrication, such as glass, for the most part these materials lack one or more of the desirable characteristics of the indicated materials and therefore have not been discussed. However, there may be particular situations where glass, ceramic or other material may find application, such as a glass window for optical clarity, modification of surface tension.

The device will normally include a reagent within the reaction chamber. In formulating the reagent(s), it may be formulated neat or with various additives. The manner in which it is formulated, introduced into the reaction chamber and maintained in the reaction chamber, must provide for rapid mixing with the sample, reproducible distribution in the chamber, stability during storage, and reproducible reaction with the sample.

In order to assure the reproducibility of distribution, various techniques may be employed for introducing the reagent into the chamber. Where the device is produced as two parts which fit together, the reagent may be sprayed, painted, introduced into the chamber as a liquid, lyophilized or evaporated, adsorbed, covalently conjugated. The active reagent may be combined with various stabilizers, excipients, buffers or other additives involved with the reaction. Alternatively, a small vial or other holder may be attached to the reaction unit, usually chamber, being stored as a liquid, where the liquid may be introduced into the reaction unit prior or concurrently with the sample entry into the reaction unit. A second receiving chamber may be employed connected to the reaction unit by a capillary channel, where transfer of the reagent in the second receiving chamber to the reaction unit is initiated in relation to introduction of the sample. For example, the second receiving chamber could be filled and sealed, and then unsealed when the sample is introduced into the sample receiving unit.

To enhance mixing, various mechanical or ultrasonic means may be employed to agitate the sample and reagents, where the mixing means may be internal or external. Vibrators, ultrasonic transducers,

magnetic rods or other mechanical mixing means, flow disrupters, mixing baffles or barriers, flow directors, may be employed. The particular manner in which agitation is provided, if provided, will vary widely depending upon the degree of agitation needed, the design of the device.

Various chemicals can be used to ehance dissolution in a uniform manner. Such chemicals may include surfactants, polyols, sugars, emollients, liquids. Depending upon the nature of the reagents, the reagents may be formulated in a variety of ways to insure rapid and uniform mixing.

Other chemicals can also be present in the reagent chambers. For example, if the device is being used to measure prothrombin time and a control sample containing heparin is being used, such as described in U.S. application serial No. 880 696, entitled "Whole Blood Control Sample", which is herein incorporated by reference, said application being assigned to the same assignee as the present application, a heparin antagonist can be used to eliminate the effects of heparin on prothrombin time measurement. Typical heparin antagonists include protamine sulfate and polybrene.

The reagent need not be coated or bound to the surface of the device, but may be provided as a soluble sponge or gel or alternatively, absorbed onto an insoluble sponge, membrane, paper (e.g., filter paper) or gel which is introduced into the reaction unit. In this manner the fluid may pass through the foam structure dissolving the reagent so as to form the reaction mixture.

The reagent may be provided in liquid form in microcapsules. The liquid reagent could be released from the microcapsules by applying pressure to the walls of the reaction unit, resulting in breaking of the microcapsules and releasing the liquid reagent.

Also, as already indicated, the reagent need not be limited to a single reaction unit. The same or different reagents may be introduced into the capillary or in successive reaction units. In this manner a cascading reaction may be performed, where one is interested in allowing each reaction step of a sequence to proceed for a predetermined period before encountering the next reagent. Multiple reaction units also allow for the removal of components in the sample which may interfere with the desired reaction. By having receptors in the first units, one or more endogenous components bay be removed. Where particles are to be removed, filters may be employed at the entrance or exit to a reaction unit.

In addition to the chemical reagent, micro-particles may be also included in the reaction unit which would be entrained with the moving front, where the microparticles could aid in the plug-forming mechanism for flow stoppage.

In performing the assay, a sample would be taken and treated as may be appropriate. Blood for example might be diluted and various reagents added, particularly where there is an interest in the determination of a particular clotting or anti-clotting factor. In specific binding assays, various particles might be added which had been functionalized by the addition of specific binding members, such as haptens, ligands, and receptors, particularly antibodies. In some instances, the system may be devised where clotting will occur in the absence of the analyte. Thus, reagents will be added which, in the absence of the analyte, would be degraded in the reaction chamber.

Once the various materials are mixed to form the sample medium, the sample medium would be introduced into the receiving unit and transferred by capillary action into the next unit. Either visual evaluation of the flow rate change or an electro-mechanical evaluation may be employed. The initiation of flow through the first capillary channel or through a successive capillary channel may be selected as the initiation time for measurement, or some point in between. As already indicated, various means may be employed for determining the flow velocity or time to flow stoppage.

For measuring a speckle pattern, which is obtained with particles, as are present in blood, an apparatus comprising a semiconductor laser and photodectors may be employed. By exposing a photodetector of sufficiently small area to a speckle pattern, a random signal (noise) is observed. The average of the random signal observed as a DC signal is inversely proportional to the red cell density, and changes in the fluctuation continues until flow stoppage, e.g., clotting, occurs. Such apparatus may include a housing for receiving and holding the device and means for controlling the temperature.

The size of the area which is detected by an individual photodetector may be controlled in a variety of ways. One way, as indicated above, is to use a photodetector which has only a small photosensitive area, up to about the size of the speckle spot. Another way is to use an optical fiber. By controlling the parameters of the fiber, the area from which the fiber receives light may be controlled. Instead of a fiber, lenses may be employed to limit the observed area which lenses may be separate from or molded into the device.

Where other than flow stoppage is involved, various spectrophotomers, fluorimeters, or the like, may be employed for detection of the detectable signal. Depending upon the nature of the assay protocol, a single determination or multiple determinations may be made, based on a fixed value or a kinetic determination.

Various devices may be devised for the subject assays. In Figures 1A and 1B devices are depicted involving single chambers and one or two capillary units. These devices can be fabricated in a variety of ways, for example, having two sheets, where each of the sheets have been molded so as to define the particular units or one of the sheets defines the units and the other is a cover sheet, or having three sheets, where a sheet having cutouts defining the units is sandwiched between the other two sheets, where one or the other sheets provides the necessary orifices for the various ports. Other techniques may also be found to be useful for providing the chamber and channel cavities.

In employing device 10 of Figure 1A, capillary 12 is introduced into the sample, so that the inlet port 14 is completely submerged in the sample. It is important to avoid any air bubbles where the air bubbles could interfere with the measurement. The inner surface of the upper portion of capillary 12 is coated with reagent 16, so that as the liquid sample transits the capillary 12, the reagent 16 becomes dissolved in the sample. When the liquid front reaches index 18, the capillary is removed from the sample, defining the sample volume, and the capillary inserted into a second fluid to maintain continuous flow. Otherwise, the sample drop can maintain a reservoir outside of the inlet port 14. The sample flows through capillary 12 into chamber 20 having vent 22. A second reagent 24 is coated onto the inner surface of reaction chamber 20, where the assay medium undergoes a second reaction.

In using this device, an assay medium could be prepared as the sample involving the fluid suspected of containing the analyte and a buffered mixture of enzyme-analyte conjugate. The reagent 16 would be antibody to the analyte, so that the enzyme-analyte conjugate present in the assay medium would become bound to the antibody in an amount related to the amount of analyte in the assay medium. The assay medium would then enter the chamber 20, where the reagent would be substrate for the enzyme. One can employ a substrate of limited solubility, so that the amount of substrate rapidly reaches equilibrium and remains constant during the measurment. One can also have a high concentration of a soluble substrate to maintain substrate concentration constant during the measurement. One can then determine the rate of formation of product which will be dependent upon the amount of active enzyme present in the chamber. Since the amount of active enzyme can be related to the amount of analyte, this rate will therefore be proportional to the amount of analyte in the sample. By employing a substrate and enzyme which produces a colored or fluorescent product, the rate can be monitored by the change in color or change in fluorescence over a predetermined time period.

In Figure 1B, device 30 has capillary 32 which is divided into channels 34 and 36 containing reagents 38 and 40, respectively. The two channels share a common inlet port 42. Channel 34 contains reagent 38, referred to as the first channel and the first reagent, which could be microparticles to which are conjugated antibodies to a first epitope. Channel 36 and reagent 40, referred to as the second channel and the second reagent, would contain microparticles having monoclonal antibodies to a second epitope. In each case, the amount of monoclonal antibody would be substantial excess of any analyte which would be encountered. The analyte of interest would have a single epitope binding to the first reagent and a single epitope binding to the second reagent. The sample would travel through the channels at a substantially constant rate, with reaction occurring with any substance having the appropriate epitope.

All of the components present in the assay medium having the appropriate epitopes would react with the particle conjugates and become bound to the particle conjugates. The particle conjugates would then exit channels 34 and 36 and enter incubation chamber 44. The chamber would also provide for capillary action, and agitation due to its accordian shape and vanes 46 for causing turbulence in the chamber 44. Thus, as the assay medium exited the channels 34 and 36, the microparticles would mix and cross link, if any analyte was present which had the two epitopes on the same molecule.

In the incubation chamber, the particles would have sufficient time to aggregate, so that upon entry into exit capillary 48, the particles which are formed would have a significant effect on the flow rate in capillary 48. By measuring the rate of flow or determining particle size or number of particles in capillary 48, one could determine the presence and amount of an analyte having both epitopes. The rate of flow or other parameter could be determined by the rate at which particles above a certain size transited a light path, using minimum light intensity fluctuations, level of scatter.

The subject device illustrates the opportunity for having a plurality of capillaries to divide a sample into a plurality or portions, where each of the portions can be treated differently. The differently treated portions may then be brought together into a single chamber, where the different portions may interact in accordance with the desired protocol. Depending upon the nature of the protocol, the resulting assay medium may then be transferred to a capillary which may provide for measurement or way be further transferred to additional chambers for further modification.

In Figures 2A and 2B are depicted a device having a plurality of chambers and allowing for interrupted flow. The device 50 is fabricated from three sheets, an upper sheet 52, a lower sheet 54, and a spacing

sheet 56, which defines the various capillary units and chamber units. The device has three chambers, the receiving chamber 58, the reaction chamber 60, and the effluent chamber 62. Inlet port 64 receives the sample which is measured by filling chamber 58 and reaching the first capillary 66. Receiving chamber 58 is coated with first reagent 68, so that the sample undergoes a reaction in receiving chamber 58 and is modified.

The modified sample then passes through capillary 66 and enters reaction chamber 60, which is coated with second reagent 70. The second reagent, like the first reagent, is part of a detection system to provide for a detectable signal. Vent 72 is provided to stop the flow and allow for incubation in reaction chamber 60. This can be particularly useful where the slow step in the development of the detection system is complex formation. Depending upon the nature of the protocol, the period of incubation may be specifically timed incubation or may be one which is allowed to sit for a sufficient time to ensure completion and then the determination made. Effluent chamber 62 has exit vent 74 to permit flow past intermediate vent 72 when intermediate vent 72 is closed. Upon closing of intermediate vent 72, the assay medium will then flow through capillary 76 into effluent chamber 62. Instead of sealing the vent, other alternatives, such as applying pressure or centrifugal force, may be used to restart flow.

The device is comprised of a block 78 which may be configured to be introduced into a instrument for assay determination. As already indicated, the various sheets will be constructed so as to ensure sufficient mechanical stability to withstand capillary action and provide for the necessary characteristics for flow of the assay medium and detection of the detectable signal.

The subject device may be employed for flow stoppage, such as coagulation, where the coagulation may occur in the effluent chamber 62. One could measure the rate of flow in capillary 66 or determine the time of flow stoppage, particularly where capillary 76 is elongated (See Fig. 5) and a plug forms in capillary 76.

In this device, one could provide for a determination of particle count, where the first chamber has bead conjugated ligands and the analyte of interest is a particular antibody. The sample would be introduced into the receiving chamber 58, where reaction would occur between the bead conjugated ligands and any antibody present in the sample. The sample would then flow to the reaction chamber 60, which would contain a reagent which binds specifically to antibody-ligand complexes, such as S. aureus protein A or rheumatoid factor, which binds specifically to poly(antibody-ligand) complexes. Thus, any bead conjugate which becomes bound to antibody, would be removed from the liquid phase of the assay medium. The device 50 could then be inserted into an instrument, which would cover vent 72, allowing for flow through capillary 76. One could then determine the number of particles or beads in the assay medium in the chamber 62 or capillary 76 as a measure of the amount of antibody in the sample.

Alternatively, one could provide for complex formation between Fab fragments and major histocompatibility antigens of cells in the receiving chamber 58. Thus, reagent 68 would be Fab fragments of monoclonal antibodies specific for the major histocompatibility antigen. The Fab fragments could be from murine or other non-human source of monoclonal antibodies. The reagent 70 would then be particles to which anti-murine immunoglobulin were conjugated. In this way, when the Fab-bound cells entered the reaction chamber 60, they would bind to the latex particle conjugates so as to form extended structures. Upon closing of the vent 72 by introducing device 50 into an instrument, the medium would flow through capillary 76, where large particles could be determined by the scattering of light, or the pattern of transmission of light through the capillary and blockage by the cellular-particle aggregations.

In Figure 3 is depicted a device which is exemplary of the determination of a plurality of anlaytes in a single sample. The device 80 has a receiving chamber 82 with inlet port 84.

The sample would be introduced into the receiving chamber 82 and be pumped by capillary action through channel 86 into reaction chamber 88. In reaction chamber 88 would be one or more reagents 108 which would provide part of the detection system. From reaction chamber 88, the assay medium would then be pumped by means of capillaries 90, 92 and 94 to chambers 96, 98 and 100, respectively. The media in chambers 96 and 98 would then be pumped by means of side capillaries 102 and 104 to final chamber 106. In this way, a variety of reactions could occur, where reagents could be provided in the various side chambers for further reaction allowing for detection of a plurality of epitopic sites.

An illustrative of the above apparatus, one could determine from a lysate, the serotype of a particular pathogen. The lysate would be introduced through inlet port 84 into the receiving chamber 82 and then be pumped into the reaction chamber 88. In the reaction chamber would be reagent 108 which would be monoclonal antibody-bead conjugates to a public epitope of the particular pathogen. One would then measure the particle count in capillaries 90, 92 and 94, which would provide the base line for the particle count which should be present in chamber 96, 98 and 100. In each of the chambers 96, 98 and 100, would be monoclonal antibodies to an epitope specific for a particular serotype, where the antibodies are

conjugated to larger beads which are distinguishable by light scattering properties from the beads in chamber 108. Thus, if the signal character changes in chambers 96, 98 or 100, this would be indicative of the particular serotype.

If one wished to determine if the serotype had another antigen of interest, one could provide for antibodies to the particular antigen in chamber 106. Chamber 106, a narrow chamber susceptible to particle blockage, would have vent 110 to allow for flow into chamber 106. Capillaries 102 and 104 would pump the assay medium from chambers 96 and 98 into chamber 106, where the presence of the particular antigen would result in cross linking of the antigen. Cross-linking of the antigen would result in plug formation.

Other combinations of labels and protocols may be employed. By dividing the assay medium into multiple pathways, the assay medium can be treated in multiple different ways and, if desired, rejoined in a single chamber as indicated above. This can be useful in situations where one is interested in different analytes, which may have different combinations of epitopes, or where the analyte must be treated in different ways in order to provide the detectable signal, or where one wished to add different combinations of labels to the analyte, where one wishes to provide a check on the results observed, or the like.

In Figure 4 is depicted a device which allows for the simultaneous determination of a sample background value and the detectable signal. The device 120 has an inlet port 122 separated by partition 124 which extends through capillary 126, chamber 128 and second capillary 130. Capillary 130 evacuates into effluent chamber 132 having vent 134. Chamber 128 is divided into two half chambers or semichambers 136 and 138. In semichamber 136, two reagents are present indicated by the slanted lines and the crosses. The slanted lines are monoclonal antibodies specific for an epitope on the analyte, where the antibodies are non-diffusively bound to the surface. The crosses indicate monoclonal antibodies conjugated to fluorescers, where the monocolonal antibodies bind to a different epitope of the analyte. The fluorescer conjugate is reversibly bound to the surface of the two chambers 136 and 138 in the area near the entry ports 140 and 142 of capillary 126.

In carrying out the assay, the sample inlet port is submerged into the sample and the sample allowed to rise in the capillary 126. Sufficient sample is introduced and the two chambers 136 and 138 are filled.

As the sample transits the two semichambers, different events will occur. In the sample reaction chamber any analyte will become bound both to thee antibody bound to the surface and the fluorescer conjugate, so that the amount of fluorescer conjugate which remains in the sample reaction chamber 136 will be dependent upon the amount of analyte in the sample. By contrast, sample which traverses control reaction chamber 138 will bind to the fluorescer conjugate but continue through the chamber into capillary 130.

By appropriate optics, one can read the fluorescence from the two capillary regions 144 and 146. The capillary region 144 will be the region for determination of the amount of analyte, while the capillary region 146 will serve as the control. Thus, the amount of fluorescence observed in region 146 will be the maximum amount of fluorescence available from the combination of sample and fluorescer conjugate. Any reduction in fluorescence in the capillary region 144 will be as a direct result of the presence of analyte. The two streams will then exit into effluent chamber 132.

As shown in Figure 5, the next embodiment 160 provides a serpentine path. The device has a housing 162 which is a rectangular plastic block shaped to fit into a reading apparatus (not shown). The block is indexed at site 164 for alignment to the apparatus. The receiving chamber 166 has a volume about one and one-half times the volume of the reaction chamber. The two chambers are connected by the first capillary channel 168. Inlet port 170 provides for introduction of the assay sample by syringe, eyedropper, or other convenient means. A serpentine capillary path 172 connects to outlet 174 of reaction chamber 176. The serpentine channel 172 terminates in reservoir chamber 178 which has outlet port 180.

Various other configurations can be employed in particular situations. For example, one could employ a "Y"-shaped device where one arm of the Y has a sample receiving chamber with the inlet port sealed. The sample receiving chamber is connected through a first conduit to the mixing member which serves as the trunk of the Y. The first conduit may be filled with a fluid which may be a buffer solution or other diluent.

The second arm of the Y has a fluid chamber with a removalbe seal over a port. The fluid chamber is filled with a fluid which may serve as a diluent, reagent source, or the like. The fluid chamber may be as large as desired so that it may provide the desired ratio of fluid to sample. The fluid chamber is connected to the mixing member through a second conduit which is also filled with the fluid of the fluid chamber. The cross-sections of the first and second conduits are selected to provide for the proper volume ratio of the fluid and sample.

The mixing member may be a capillary or chamber. The mixing member may be the final element of the device or may be connected to additional elements. Thus, the Y can serve as a complete device, by providing the mixing member with an outlet port or a part of a larger device by connecting the mixing

member to additional capillaries and/or chambers.

To use the Y, the seals are removed from the fluid chamber and sample chamber, while retaining the outlet port sealed to prevent flow. The inlet port may then be contacted with the sample and the outlet port unsealed. The sample and fluid from the two chambers will begin to flow and be mixed in the mixing member in the proper proportion. Depending on the particular protocol, one can determine a number of different events by reading the flow of fluid in one of the capillaries.

One application would be to provide fluorescent antibodies in the fluid chamber. Where the sample contains cells having the homologous ligand the mixing of the fluorescent antibodies and cells will result in large fluorescent particles as a result of the homologous antigen being present on the cells. These large fluorescent particles could then be detected by various means.

Figure 6 shows a cross-sectional view through a section of a hypothetical device being prepared by ultrasonic welding from two injection-molded plastic pieces (not necessarily shown in any of the previous figures), prior to ultrasonic welding. The two plastic pieces 202 and 204 that will be used to form the internal chambers are shown properly aligned (i.e., in register) in Figure 6A. "Registration" is used here in the printing sense, referring to proper alignment of the depressions present in the surfaces of the two pieces that are used to form the internal chambers and capillaries. Proper registration can be aided by injection molding the two pieces to provide projections on one piece that fit into holes or depressions (other than capillary- or chamber- forming depressions) in the second piece. A single convoluted depression, 206 and 208, respectively, is formed into the surface of each piece, but the cross-sectional view shown in the figure cuts through the depression at three separate locations, two of which will result in capillary spaces (210 in Figure 6B) while the remaining location will result in the formation of a larger reaction chamber (212 in Figure 6B). Energy-directing ridges 214 can be seen in the surface of one of the two plastic pieces (204) adjacent to the periphery of the depression.

Figure 6B shows the same cross-sectional view after ultrasonic welding. The plastic has melted selectively in the region of the energy-directing ridges so that the two plastic pieces have melted into each other to form a seal around the capillary and the reaction chamber. In order to minimize the destructive effects of heat caused by the ultrasonic welding, ultrasonic welding is carried out only until a seal is formed and does not need to be carried out until the entire plastic surfaces have welded together. Unwelded contact surfaces are shown by reference numbers 216 in Figure 6B. Use of energy ridges and short welding times also ensure that the dimensions of the depressions will be unaffected by the welding event. Welding time will be selected so that the melting (welding) almost but not quite reaches the edge of the depression. The extremely small cracks left between the two plates in the area of the capillaries will not adversely effect capillary action.

Figure 7 shows electronic circuitry that can be utilized to simulate the passage of blood through a capillary flow device. The circuitry includes a crystal-controlled oscillator in which 220 represents the crystal and 222 represents the oscillator. The signal from the oscillator drives two frequency dividers (224 and 226) that will generate the output signals for a divider 228 of a liquid crystal display cell 230. Cell 230 is biased by an oscillating signal having a specific rate of oscillation, for example 128Hz. The cell will therefore rotate its polarization at the rate of 128Hz. Polarizer 231 in combination with cell 230 therefore operate to alternately block and pass light as a result of the rotating polarization.

Two more dividers (232 and 234) drive a logical AND gate (236) whose output will go to a logic circuit low at defined intervals, for example, approximately every 20 seconds. When the output goes to a logical low, the output of a logical OR gate (238) will reset the dividers, thereby stopping the process. Accordingly, modulating signals for the liquid crystal display cell 230 are generated for the set time period, 20 seconds in the above example.

The device is provided with a start switch 240. When switch 240 is closed, the reset signal is cleared, and the process is restarted.

The oscillator, dividers, logic gates, and liquid crystal display driver can be implemented in CMOS technology using standard techniques of electronic fabrication. An CMOS device can be readily powered through more than 10,000 cycles when powered by a coin-type lithium battery.

Figure 8 shows a device for determining when the sample present in a reservoir is depleted. In this figure, blood is presumed to be the sample. When blood is applied to sample reservoir 242 it will start flowing immediately down capillary channel 254 by capillary action. A light source 246, typically an infrared light-emitting-diode (LED), is located adjacent to the blood reservoir near the capillary entrance. Light source 246 is modulated by a modulator 248, typically a sinusoidal wave or square wave generator. A typical modulation frequency is about 8KHz. The modulated light will be scattered by red blood cells in the blood sample, and a fraction of this light will be guided by capillary channel 254 formed in the plastic capillary flow device 250. Since the red blood cells in the sample will further scatter this guided light,

photodetector 252, typically located in close proximity to the inlet 254 of the capillary channel, will capture some of the scattered light. The signal output of the photodetector will consist of the superposition of ambient light and scattered light. The scattered light component is separated from the ambient light by a band-pass filter 256 and is further amplified by amplifer 258. This signal is rectified by rectifier 260 and integrated by integrater 262 in order to generate a direct current voltage proportional to the scattered light. Other types of signal generators can be used to produce a detectable signal that is seperable from ambient light and its possible variations. Examples include wave lengths (e.g., use of infrared light sources), light pulses, sinusoidal wave generation, and digital encodation. When methods other than frequency modulation are used to produce the detectable signal, the term "filter" as used in this specification refers to any means of separating the detectable signal from variation in ambient light.

Although the exact location of the ambient light source and detector in relation to the junction between the blood reservoir and the capillary can be varied depending on the capillary size, strength of a light source, detection limit of the detector, adsorbance of the sample, it is preferred that both the light source and the detector by relatively close to the junction, particularly when a highly abosrbant sample such as blood, it is utilized. When an infrared light source is used, it is preferred to place the light source from 0.5 to 2mm from the capillary entrance with an infrared-sensitive photodetector being located from 1 to 4mm from the junction.

As the blood reservoir empties due to the capillary flow, the light path between light source 246 and photodetector 252 will be interrupted, thereby reducing the voltage output from integrator 262. By connecting the output of the integration to a comparator 264, a logical level indicating the presence or absence of sample in the reservoir is obtained. Furthermore, by adjusting the position of the light source or the reference voltage of the comparator, the volume of sample in the reservoir at which the decision "no sample" is made can be controlled.

An infrared light source is preferred because of its larger efficiency in converting electric current into light, as compared to visible-light light-emitting-diodes. A modulating frequency of several kilohertz (preferably 3 to 20 kHz) is selected in order the move the modulation frequency from the low 60Hz harmonics present in artificial illumination, thereby simplifying the separation of ambient light and signal light. The separation is enhanced even further by the selection of an infrared light source.

Obviously, various designs of the Individual chambers and channels can be provided. The designs and channels will be selected to provide for optimum sensitivity for particular assays. The volumes of the chambers will be chosen so as to accommodate an appropriate sample volume. The nature and cross section of the first capillary channel together with the size of the reaction chamber will control the residence time of the assay medium in the reaction chamber. In some systems a reaction will terminate upon the sample exiting the reaction chamber, e.g., antigen-antibody complex formation, pro-enzyme to enzyme, etc., where a component is bound to the surface of the chamber. The reaction occurring in the reaction chamber, may result in a product which produces a blockage in the second capillarly channel or prevents a blockage from forming. The residence time for the reaction in the reaction chamber can be carefully controlled by controlling the dimensions of the capillary channels and reaction chamber, as well as temperature.

It is evident that any type of capillary channel may be employed which provides for accommodating the appropriate volume and time period to flow stoppage. Various designs may be used such as serpentine, linear, U-shaped, pleated, or the like. The channel cross-section may be circular, ellipsoid, rectangular, or combinations thereof. The length of the channels may be determined empirically depending upon the other parameters involved.

The initial or metering channel may be of constant or varying cross-section. With a constant cross-section, the observed flow velocity will diminish with the path length traversed. Therefore, the observed change in velocity will have two components: (1) an inherent reduction in velocity related to the increasing friction with increasing fluid path length; and (2) increasing or decreasing viscosity of the medium due to any reaction occurring.

In order to eliminate the effect of the fluid path length, a tapered capillary may be employed. The taper can be calculated by determining the cross-section, e.g., height and width, of the channel for each point along the channel path. The equations below are employed. The equations are based on known principles of fluid mechanics (e.g., R. Byron Bird, Warren E. Stewart, Edwin N. Lightfoot, Transport Phenomena, John Wiley & Sons, Inc., 1960).

The flowrate, Q, measured by the laser (in the first capillary channel) can be defined by:

$$Q = VA = \frac{\pi r^4}{8\mu} \cdot \frac{\Delta P}{z} \qquad (1)$$

where, V is the velocity in the 2nd capillary, and A is the area of the 1st capillary channel, and:

- $r$ = radius of the 2nd capillary channel
- $\mu$ = viscosity of the fluid
- $z$ = distance down the 2nd capillary channel
- $\Delta P$ = the pressure drop in the 2nd capillary

is defined by:

$$\Delta P = \frac{2\gamma \cdot \cos \phi}{r} \qquad (2)$$

wherein:

- $\gamma$ = surface tension of the fluid
- $\phi$ = contact angle of fluid with surface

Combining (1) and (2), the flowrate, Q, becomes:

$$Q = \frac{\gamma r^3 \pi \cdot \cos \phi}{4z\mu} \qquad (3)$$

From (3) it can be seen that the radius of the 2nd capillary channel is proportional to the distance down the channel, z:

$$r = kz^{1/3}$$

where

$$k = \frac{4Q^*\mu}{\gamma\pi \cdot \cos \phi}$$

and

$Q^*$ is the desired constant flowrate in the 2nd capillary channel.

Using the above equations and selecting a desired $Q^*$, the following table indicates the changes in radius at the position defined by z, for k = 0.034

| Length mm | Radius mm | Flowrate $mm^3/sec$ | Inc Vol $mm^3$ | Vol $mm^3$ | Flow Time sec |
|---|---|---|---|---|---|
| 5 | 0.090 | 0.206 | 0.13 | 0.127 | 0.62 |
| 10 | 0.090 | 0.103 | 0.13 | 0.254 | 1.85 |
| 15 | 0.090 | 0.069 | 0.13 | 0.382 | 3.70 |
| 20 | 0.092 | 0.056 | 0.13 | 0.515 | 6.11 |
| 25 | 0.099 | 0.056 | 0.16 | 0.671 | 8.91 |
| 30 | 0.106 | 0.056 | 0.18 | 0.846 | 12.06 |
| 35 | 0.111 | 0.056 | 0.19 | 1.040 | 15.56 |
| 40 | 0.116 | 0.056 | 0.21 | 1.253 | 19.38 |
| 45 | 0.121 | 0.056 | 0.23 | 1.482 | 23.51 |
| 50 | 0.125 | 0.056 | 0.25 | 1.729 | 27.95 |
| 55 | 0.129 | 0.056 | 0.26 | 1.992 | 32.68 |
| 60 | 0.133 | 0.056 | 0.28 | 2.270 | 37.68 |
| 65 | 0.137 | 0.056 | 0.29 | 2.563 | 42.97 |
| 70 | 0.140 | 0.056 | 0.31 | 2.872 | 48.52 |
| 75 | 0.143 | 0.056 | 0.32 | 3.195 | 54.33 |
| 80 | 0.147 | 0.056 | 0.34 | 3.532 | 60.40 |
| 85 | 0.149 | 0.056 | 0.35 | 3.883 | 66.72 |
| 90 | 0.152 | 0.056 | 0.36 | 4.248 | 73.28 |
| 95 | 0.155 | 0.056 | 0.38 | 4.626 | 80.08 |
| 100 | 0.158 | 0.056 | 0.39 | 5.017 | 87.12 |
| 105 | 0.160 | 0.056 | 0.40 | 5.421 | 94.40 |
| 110 | 0.163 | 0.056 | 0.42 | 6.838 | 101.90 |
| 115 | 0.165 | 0.056 | 0.43 | 6.267 | 109.63 |
| 120 | 0.168 | 0.056 | 0.44 | 6.709 | 117.58 |
| 125 | 0.170 | 0.056 | 0.45 | 7.163 | 125.75 |
| 130 | 0.172 | 0.056 | 0.47 | 7.629 | 134.13 |
| 135 | 0.174 | 0.056 | 0.48 | 8.107 | 142.73 |

| Length mm | Radius mm | Flowrate $mm^3$/sec | Inc Vol $mm^3$ | Vol $mm^3$ | Flow Time sec |
|---|---|---|---|---|---|
| 140 | 0.177 | 0.056 | 0.49 | 8.596 | 151.55 |
| 145 | 0.179 | 0.056 | 0.50 | 9.098 | 160.56 |
| 150 | 0.181 | 0.056 | 0.51 | 9.610 | 169.79 |
| 155 | 0.183 | 0.056 | 0.52 | 10.134 | 179.22 |
| 160 | 0.185 | 0.056 | 0.54 | 10.669 | 188.85 |

It is evident from the above table that the path may change from a capillary of constant radius, to one in which the radius increases (e.g., a funnel) or decreases with distance. This formula can be used to vary velocity as desired as the liquid moves down the capillary track or through a region of reagent. On could even envision a pulsating flow.

In accordance with the subject invention, novel devices and methods are provided for measuring a wide variety of sample characteristics including analytes of interest. The devices provide for simple measurement or volumes, mixing of reagents, incubations, and visual or instrumental determination of the result. The detection system may involve the absorption or emission of light, or modulation of flow, including slowing, stoppage, or their reversal. Of particular interest is the use of blood where clotting can be determined or reagents affecting clotting. Also of interest are a wide variety of analytes, which include naturally-occurring compounds or synthetic drugs. The devices allow for the simultaneous performance of controls and comparison of signals from the two media. In addition, various combinations of channels and chambers may be employed, so that the pathways can diverge and converge, be broken up into a plurality of different pathways or a sample may be divided into a plurality of paths and treated in a variety of ways. The devices can be simple to fabricate and the serpentine paths readily devised by employing known fabrication techniques, with particularly advantageous devices being available through use of the preferred fabrication techniques described herein.

The following examples are offered by way of illustration and not by way of limitation.

## EXPERIMENTAL

### EXAMPLE 1: Detection of Prothrombin Time

A device or cartridge analogous to the device of Figure 5 is employed. Two pieces of cellulose acetate 0.2032 mm (40 mil) thick are separated by scored Fason fast tape B to provide the proper design of channels and chambers. The reaction chamber contains a thromboplastin reagent where an aqueous solution of the thromboplastin is introduced into the chamber, the water evaporated, leaving the chamber coated with thromboplastin. The thromboplastin reagent composition is: 15mg/ml rabbit brain thromboplastin extract; 1% glycine; 0.01% thimerosol; 0.01% streptomycin-sulfate; 0.01% triton X100$^R$; 0.08% phenol; 1% polyethylene glycol 3500; 4% sucrose; 0.001% polybrene™. The mixture is lyophilized and reconstituted to 0.25 original volume with deionized water. Three µl of the reconstituted liquid is then placed on the reagent area of the device and then allowed to air dry before attaching the other sheet or cover of the device. A cover is then placed over the chambers and channels, and a 15µl blood sample is introduced into the receiving chamber. The device is then inserted into a monitor which may be thermostatted at 25°C or 37°C, where a gallium arsenide semiconductor laser of wavelength 0.78µ directs a beam at a site between the ends of the channel joining the receiving chamber and the reaction chamber. On the opposite side from the laser is a silicon photodetector of a small area sufficient to detect the oscillating speckled pattern resulting from the red blood cells flowing through the channel. A DC signal is observed with large fluctuations. The DC signal is inversely proportional to the red cell density, and fluctuation continues until clotting occurs. The time is then related to known standards to determine the clotting characteristic of the blood. Where warfarin is being administered to the blood source, the time for clotting can be related to the effect of warfarin on the blood clotting time. In addition, the light absorbance in the channel can be determined to provide for a hematocrit, as a further characteristic of the blood sample. Because of the short path length of the light, undiluted blood can be used which provides a further convenience.

EXAMPLE 2: Detection of Cross-linked Fibrin Dimer by Latex Aggultination

Materials: "Dimertest latex" reagents and control serum (MABCO Ltd). A cartridge analogous to that of fig. 5 is used. Two flat pieces of polystyrene formed by injection moulding are welded together to form the capillary channel and reagent chamber. The receiving chamber (166) is 0.467cm wide by 0.09cm deep. The first capillary channel (168) is 1.3mm wide by 0.09mm deep. The reagent chamber (176) is a elipse of major axis 12mm and minor axis 6mm and is 0.09mm deep. The serpentine capillary path (172) is 160mm long tapering from a radius of 0.09mm at the outlet or the reagent chamber to 0.185mm at the outlet port (180).

Experimental: Sample (10µl of either buffer or positive control) was mixed with antibody coated latex (40µl); after 3' gentle shaking 40µl of the mixture was injected into the cartridge containing no reagent and inserted into a monitor as described in Example 1. The signal from the laser detector was recorded on an Omega chart recorder (model 1202) using 5V full scale sensitivity and a chart speed of 6cm/min. The movement of latex particles through the light beam produced a slightly noisy trace (~1 chart division {0.01 full scale} peak-peak). Agglutination of the latex caused by the presence of the analyte (cross-linked fibrin degradation products) resulted in a significant increase in noise (~3 chart divisions peak-peak).

EXAMPLE 3: Direct Blood Grouping by Red Blood Cell Agglutination

Materials: Human blood samples of known groups anticoagulated with sodium citrate. Blood grouping antisera (American Dade).

Experimental: Resuspended blood (40µl) was mixed with antiserum (40µl) and 40 µl of the mixture injected into empty cartridges (as in example 2) and results analyzed as in Example 2. Positive agglutination was observed as a rapidly increasing noise level, negative reactions gave a steady, low, noise level.

EXAMPLE 4: Direct blood grouping by flow stop

Materials: Blood samples as in Example 3. Cartridges as described in Example 2 were employed. Before welding the two parts of the cartridge, the serpentine track of the lower part was evenly coated with 5µl blood typing anti-serum (American Dade) which had been dialysed against 1% glycine (Na+) pH 7.5 containing 0.01% Triton X-100®, 2% sucrose and 0.5% polyethylene glycol -3500. Solvent (water) was then removed by evaporation and the two parts of the cartridge welded together.

Experimental: Blood (40µl) was injected into the cartridges after insertion in a monitor (described in example 1). The time for flow of red blood cells past the laser beam to stop was recorded.

| Blood # | Group | Reagent | Time (s) | Positive or Negative* |
|---|---|---|---|---|
| 1 | A | A | 43 | + |
| | | B | 175 | - |
| 3 | A | A | 69 | + |
| | | B | 190 | - |
| 4 | B | A | >270 | - |
| | | B | 58 | + |
| 8 | O | A | >200 | - |
| | | B | >187 | - |

*A positive reaction was defined, as Time < 120s.

Agglutination of red blood cells at the leading edge of the blood caused clogging of the track and flow stop. All the blood samples gave the appropriate reaction.

EXAMPLE 5: Blood grouping by flow rate measurements

Materials: Blood samples as in Example 3; Cartridges (as described in example 2) with 3µl American Dade blood typing antisera applied (as described in example 4) to the reagent chamber and dried.

Experimental: Blood (50µl) was injected into the cartridges at room temperature. The time taken for the blood to reach known distances along the narrow track was recorded. Flow rates were then calculated from

a knowledge of the cross section of the track as a function of distance.

| Blood Sample # | Group | Reagent | Flow Rate at 150s($mm^3/s$) | Positive or Negative |
|---|---|---|---|---|
| 6 | B | A | 0.045 | - |
| | | B | 0.025 | + |
| 1 | A | A | 0.018 | - |
| | | B | 0.045 | + |
| 8 | O | A | 0.047 | - |
| | | B | 0.047 | - |

*A positive reaction was taken as flow rate <0.030 $mm^3/s$.

Agglutination of red blood cells caused a significant reduction in flow rates.

EXAMPLE 6: Use of filters to modify sample composition

a. Red cells were quantitatively removed from whole blood by filtration through a dry filter paper disc impregnated with anti human red cells. Discs were cut to fit the sample application site of cartridges from Beckman Paragon$^R$ electrophoresis blotter paper (about 0.57mm thick). A metal punch of diameter 0.467cm was used. After the discs were snugly inserted in the sample cite of the track, 10µl rabbit anti-human red cells (Cappel) was added. This is enough liquid to saturate the paper. The liquid was then evaporated under vacuum.

When blood (40µl) was applied to the disc about 7µl clear plasma emerged into the track before flow stopped.

b. Filter paper disc (as above) impregnated with sodium deoxycholate (10µl x 10%) were dried under vacuum and placed in the sample application site. Blood (40µl) was applied at 37°C. A clear uniform red solution free of all red cells filtered into and filled the track. The absorbance of the remaining hemolysate can give an accurate hemoglobin concentration.

EXAMPLE 7: Electronic Cartridge

An electronic cartridge capable of simulating the flow of whole blood through a capillary was prepared using a 32768Hz crystal-controlled oscillator to drive two 1-16th frequency dividers that generated the input signals for a driver of a liquid crystal display cell prepared in accordance with the electronic diagram set forth in Figure 7. This cell was biased by a 2048Hz signal modulated at 128Hz intervals. The cell therefore rotated its polarization at the rate of 128Hz.

Two more dividers drove a logical AND gate whose output was to a logic low every 20 seconds. At this point, the output of a logical OR gate reset the dividers, stopping the process. Accordingly, the LCD was powered and modulating for a 20-second interval.

A start switch was provided to clear the reset signal and restart the process. The oscillators, dividers, logic gates, and LCD driver were implemented in CMOS technology and powered by a coin-type lithium battery. The electronic cartidge has a life of more than 10,000 cycles.

EXAMPLE 8: Out-of-Blood Detector

The following sample depletion device was prepared in the manner shown in Figure 8. An infrared LED was embedded into the surface of the analytical device into which a capillary flow cartridge was to be inserted so that the output of its light would impinge upon the blood reservoir 1mm from the capillary entrance. The LED is powered by an operational amplifier configured as a square wave generator of about 8KHz. An infrared-sensitive photodetector was located 2mm from the junction of the sample reservoir and the capillary channel. The signal output of the detector was passed to a band-pass filter having a center at 8KHz and a bandwidth of 500Hz and was further amplified by an amplifier. The amplified signal was rectified and integrated, thereby generating a direct current voltage proportional to the scattered light.

When a blood sample is added to the reservoir and flows by capillary action through the capillary into other portions of the test cartridge, the light path between the LED and the photodetector is interrupted when the reservoir empties. When the voltage output of the integrator falls below 50-30 millivolt, the input of

this voltage into a comparator will indicate the absence of blood in the blood reservoir.

EXAMPLE 9: Fabrication

Plastic cartridges were injection molded out of Cycolac$^{R}$ CTB Resin (acrylanitrile-butadiene-styrene copolymer) obtained from Borg-Warner Chemicals, Inc. The design of the cartridge capillary channel and overall structure is similar to that shown in Figure 5 in that it contains a sample reservoir, an initial short capillary, a reagent chamber and a second long capillary. However, the configuration of the actual chambers and capillaries differ from those shown. The cartridge consisted of a 0,762 mm (30-mil) base and a 0,762 mm (30-mil) cover. Both the cover and base were plasma etched in an LFE Model 1002 Plasma system with the following settings: argon pressure, 266,6 Pa (2 torr); argon flow, 1-3; forward RF powere, 100; etch time, 20 minutes. Three microliters of Biotrack thromboplastin reagent were then applied to the base of the oval area at the base of the cartridge and allowed to dry for 10 minutes in an environment kept at 25 °C and 10% relative humidity. The etched cover was then placed on the base and welded thereto using a Branson 8400Z ultrasonic welder with the following settings: down speed, 3 seconds; hold time, 0.5 seconds; weld energy, 0.5Kjoule; and weld time, 0.26-0.30 second.

Following fabrication, the cartridges were tested with whole blood controls (both abnormal and normal clotting times), which are described in a copending application filed on even date with this application entitled "Whole Blood Control Sample" using the Biotrack Model Monitor 1000. The prothrombin times were recorded. In addition, the flow rate of blood in a cartridge without reagent was measured.

The results of the test were subjected to statistical analysis. When replicates were run, the mean and coefficient of variation were 12.3 seconds and 4.9% for the normal control and 20.0 seconds and 2.9% for the abnormal control. The flow rate of blood in the capillary channel four days later was constant at 0.06mm$^3$/sec. These results are quite superior to other fabrication techniques described in this specification, including the example using tape set forth in Example 1, which gave C.V.s in the 10-20% range.

**Claims**

1. A method for determining the presence of an amount of an analyte in, or a property of, a fluid sample comprising:
   applying said sample to a device (10) comprising an entry port (14) for said sample, a vent (22), a capillary pathway containing a chamber (12, 20) connecting said entry port (14) to said vent (22), and a reagent (16, 24) in said capillary pathway (12, 20), wherein said sample flows through said capillary pathway (12, 20) under capillary forces and interaction of said reagent (16, 24) with said sample modifies viscosity of said sample or a characteristic of said sample associated with said flow;
   allowing said sample to interact with said reagent (16, 24) and traverse at least a portion of said capillary pathway (12, 20);
   detecting said viscosity or flow characteristic; and
   relating said viscosity or flow characteristic to the presence or amount of said analyte in or, to said property of, said fluid sample.

2. The method of claim 1, wherein either said sample or said reagent (16, 24) comprises particles and said flow characteristic comprises passage of particles through said capillary pathway (12, 20).

3. The method of claim 1 or 2, wherein said flow characteristic comprises a change in particle size distribution.

4. The method of anyone of the claims 1 to 3, wherein said change in particle size distribution results from particle agglutination.

5. The method of anyone of the claims 1 to 4, wherein red cells in a whole blood sample function as particles in said capillary pathway (12, 20) and said flow characteristic comprises interruption of a light beam directed through said capillary pathway (12, 20) by said red cells as said red cells pass through said capillary pathway (12, 20).

6. The method of anyone of claims 1 to 5, wherein particles are present in said sample.

7. The method of anyone of claims 1 to 5, wherein particles are present in said reagent (16, 24).

8. The method of anyone of claims 1 to 7, wherein said flow characteristic comprises a change in rate of passage of particles through said pathway (12, 20).

9. The method of anyone of claims 1 to 7, wherein said flow characteristic is a change in flow rate of sample through said capillary pathway (12, 20).

10. The method of anyone of claims 1 to 9, wherein said sample comprises whole blood.

11. The method of anyone of claims 1 to 10, wherein said flow change is flow slowing or cessation as a result of clot formation.

12. The method of anyone of claims 1 to 10, wherein said flow change is starting flow as a result of clot dissolution.

13. The method of claim 1, wherein said sample comprises whole blood and said reagent (16, 24) induces clotting of said sample.

14. The method of claim 1, wherein said capillary pathway (12, 20) comprises at least one chamber (20) containing said reagent (24) and at least one capillary unit (12) having a cross sectional area less than the cross sectional area of said chamber unit (20).

15. The method of claim 14, wherein said capillary pathway comprises said inlet port, a chamber unit, a first capillary unit connecting said inlet port to said chamber unit, a vent, and a second capillary unit connecting said chamber unit to said vent, wherein a sample applied to said entry port is transported by said first capillary to said chamber unit and is transported by said second capillary unit from said chamber unit toward said vent.

16. The method of claim 15, wherein said device has a third port proximal to a connection of said chamber to said second capillary unit, wherein transmit of said sample terminates at said third port to permit an incubation of said sample with said reagent, and said method further comprising the step of sealing said third port after said incubation to permit continued movement of said sample from said chamber unit into said second capillary unit.

17. The method of claim 1, wherein more than one capillary pathway receives sample from a single entry port.

18. The method of claim 17, wherein each of said capillary pathways contains a different reagent.

19. A control device capable of detecting the depletion of a particle-containing fluid in a device as described in claim 1 containing a sample reservoir (242) and said capillary pathway (254) exiting said reservoir (242), which comprises:
a light source (246) located so as to impinge on said fluid in said reservoir (242);
a light detector (252) located in close proximity to said capillary pathway (254) so as to collect light which is reflected by said particles into said capillary pathway (254) and thereafter further reflected by said particles so as to pass out through the walls of said capillary pathway (254);
a signal generator (248) operably attached to said light source (246), wherein a detectable signal is imposed on the output of said light source; and
a filter (256) operably attached to the output of said light detector (252), wherein said detectable signal is isolated from other light sources which may impinge upon said light detector (252).

20. The control device of claim 19, wherein said light source (246) produces infrared light and said detectable signal is a periodic variation in the intensity of said light.

21. The control device of claim 19, wherein light from said light source (246) impinges on said fluid in said reservoir (242) at a right angle to a line formed by said capillary pathway (254).

22. A control device of claim 19, wherein said light source (246) is an infrared light source, said capillary pathway (254) is joined to said reservoir (242) at a capillary entrance, said light source (246) is located

from 0.5 to 2 mm from said capillary entrance, and said light detector (252) is located from 1 to 4 mm from said capillary entrance.

23. The control device of claim 19, wherein light from said light source (246) entering said sample reservoir (242) is reflected at essentially a right angle from said light source (246) into said capillary pathway (254) and exits said capillary pathway (254) at essentially right angle to impinge on said detector (252).

24. An analytical device for detecting the presence of an analyte in a physiological fluid sample, comprising:
a housing (162) containing an inlet port (170), a chamber unit (176), an exit port (180), a first capillary unit (168) for independently pumping a liquid from said inlet port (180) to said chamber unit (176), and a second capillary unit (172) positioned between and operatively connected to said chamber unit (176) and said exit port (180) for independently pumping a liquid from said chamber unit (176) to said exit port (180); wherein said inlet port (170), first capillary unit (168), chamber unit (176), second capillary unit (172), and exit port (180) are present in a continious capillary pathway; said housing (162) further containing a reagent in said capillary pathway comprising a member selected from the group consisting of compounds effecting blood clotting and antibodies capable of causing a change in flow rate of said fluid sample in said pathway.

25. The device of claim 24, wherein said reagent comprises an antibody.

26. The device of claim 24, wherein said device comprises at least two chamber units in said pathway.

27. The device of claim 24, further comprising a sealable vent in said capillary pathway proximal to a juncture between a chamber unit and a capillary unit.

28. The device of claim 24, further comprising a third capillary unit substantially in parallel with said first capillary unit and directing fluid into said chamber unit.

29. The device of claim 24, wherein said reagent comprises a member selected from the group consisting of components of natural blood clotting cascades and reagents which activate a component of a natural clotting cascade.

30. The device of claim 24 or 29, wherein said reagent is thromboplastin.

31. The device of claim 24, wherein said housing comprises a substantially hydrophobic material having at least a portion of the walls of said capillary pathway treated to provide hydrophilicity.

32. The device of claim 24 or 31, wherein said material is a plastic.

33. The device of claim 24, said device being fabricated from at least two sheets of a substantially hydrophobic plastic, at least one of which is molded to provide depressions for production of said capillary and chamber units, said depressions being treated to provide hydrophilicity, wherein at least one sheet has means defining an orifice forming said inlet port or exit port, said capillary and chamber units being formed by the joining of said sheets to form said housing.

34. The device of claim 24 or 33, wherein said chamber unit contains said reagent and said reagent comprises a member selected from the group consisting of components of natural clotting cascades and reagents which activate a component of a natural clotting cascade.

35. The device of anyone of the claims 24, 33 or 34, wherein said reagent comprises thromboplastin in said chamber unit.

36. A device for use in a system for detecting the presence of an analyte in a blood sample, comprising:
a device as described in claim 1 wherein said capillary pathway comprises a substantially hydrophobic housing having at least a portion of the walls treated to provide hydrophilicity, and being capable of pumping blood as the sole motive source in said device; and
a reagent in said pathway, wherein said reagent causes blood to clot and is a member of a system

capable of providing a detectable signal in relation to the presence of said analyte or property.

37. A device according to anyone of claims 24 to 36, characterized in that the reagent is fixed to the walls of said capillary pathway.

**Patentansprüche**

1. Verfahren zur Bestimmung der Anwesenheit einer Menge eines Analyten in, oder der Eigenschaft, einer Probe eines Fluids, mit den Schritten:
Einbringen der Probe in eine Einrichtung (10) mit einer Eintrittsöffnung (14) für die Probe, einer Entlüftungsöffnung (22), einem eine Kammer (12, 20) enthaltenden, die Eintrittsöffnung (14) mit der Entlüftungsöffnung (22) verbindenden Kapillarkanal (12, 20), wobei die Probe durch den Kapillarkanal (12, 20) unter Kapillarkräften strömt und die Wechselwirkung des Reagens (16, 24) mit der Probe die Viskosität der Probe oder eine Eigenschaft der Probe, die mit dem Strom in Beziehung steht, verändert;
gegenseitiges Einwirken der Probe und des Reagens (16, 24) und Durchfließen mindestens eines Teils des Kapillarkanals (12, 20);
Bestimmung der Viskosität oder der Strömungseigenschaft; und
Herstellung einer Beziehung zwischen der Viskosität oder Strömungseigenschaft zu der Anwesenheit oder der Menge des Analyten in, oder der Eigenschaft, der Probe des Fluids.

2. Verfahren nach Anspruch 1, bei dem entweder die Probe oder das Reagens (16, 24) Teilchen umfaßt und die Strömungseigenschaft den Durchgang der Teilchen durch den Kapillarkanal (12, 20) umfaßt.

3. Verfahren nach Anspruch 1 oder 2, bei dem die Strömungseigenschaft eine Veränderung der Teilchengrößenverteilung umfaßt.

4. Verfahren nach einem jeden der Ansprüche 1 bis 3, bei dem die Veränderung in der Teilchengrößenverteilung von einer Agglutination der Teilchen herrührt.

5. Verfahren nach einem jeden der Ansprüche 1 bis 4, bei dem rote Blutkörperchen in einer Vollblutprobe als Teilchen in dem Kapillarkanal (12, 20) wirken und die Strömungseigenschaft die Unterbrechung eines durch den Kapillarkanal (12, 20) gerichteten Lichtstrahls durch die roten Blutkörperchen umfaßt, wenn die roten Blutkörperchen durch den Kapillarkanal (12, 20) gehen.

6. Verfahren nach einem jeden der Ansprüche 1 bis 5, bei dem Teilchen in der Probe vorhanden sind.

7. Verfahren nach einem jeden der Ansprüche 1 bis 5, bei dem Teilchen in dem Reagens (16, 24) vorhanden sind.

8. Verfahren nach einem jeden der Ansprüche 1 bis 7, bei dem die strömungseigenschaft eine Veränderung in der Geschwindigkeit des Durchgangs der Teilchen durch den Kanal (12, 20) umfaßt.

9. Verfahren nach einem jeden der Ansprüche 1 bis 7, bei dem die Strömungseigenschaft eine Veränderung in der Strömungsgeschwindigkeit der Probe durch den Kapillarkanal (12, 20) ist.

10. Verfahren nach einem jeden der Ansprüche 1 bis 9, bei dem die Probe Vollblut umfaßt.

11. Verfahren nach einem jeden der Ansprüche 1 bis 10, bei dem die Strömungsveränderung eine Strömungsverlangsamung oder Strömungsunterbrechung als Ergebnis der Bildung eines Gerinnsels ist.

12. Verfahren nach einem jeden der Ansprüche 1 bis 10, bei dem die Strömungsveränderung das Einsetzen der Strömung als Ergebnis der Auflösung eines Gerinnsels ist.

13. Verfahren nach Anspruch 1, bei dem die Probe Vollblut umfaßt und das Reagenz (16, 24) die Gerinnung der Probe induziert.

14. Verfahren nach Anspruch 1, bei dem der Kapillarkanal (12, 20) mindestens eine das Reagenz (24) enthaltende Kammer (20) und mindestens eine Kapillareinheit (12) mit einer Querschnittsfläche kleiner als die Querschnittsfläche der Kammereinheit (20) umfaßt.

15. Verfahren nach Anspruch 14, bei dem der Kapillarkanal die Eintrittsöffnung, eine Kammereinheit, eine erste Kapillareinheit, die die Eintrittsöffnung mit der Kammereinheit verbindet, eine Entlüftung und eine zweite Kapillareinheit, die die Kammereinheit mit der Entlüftung verbindet, umfaßt, wobei eine in die Eintrittsöffnung gebrachte Probe durch die erste Kapillar- zu der Kammereinheit und durch die zweite Kapillareinheit aus der Kammer zu der Entlüftung transportiert wird.

16. Verfahren nach Anspruch 15, bei dem die Vorrichtung eine dritte Öffnung proximal zu einer Verbindung der Kammer mit der zweiten Kapillareinheit aufweist, wobei der Durchtritt der Probe an der dritten Öffnung endet, um eine Inkubation der Probe mit dem Reagenz zu gestatten, und wobei das Verfahren weiterhin einen Schritt umfaßt, gemäß dem die dritte Öffnung nach der Inkubation verschlossen wird, um eine fortgesetzte Bewegung der Probe von der Kammereinheit in die zweite Kapillareinheit zu gestatten.

17. Verfahren nach Anspruch 1, bei dem mehr als ein Kapillarkanal eine Probe von einer einzigen Eintrittsöffnung erhält.

18. Verfahren nach Anspruch 17, bei dem jeder der Kapillarkanäle ein unterschiedliches Reagenz enthält.

19. Kontrolleinrichtung zur Bestimmung der Entleerung eines partikelhaltigen Fluids in einer Vorrichtung nach Anspruch 1, enthaltend ein Probenreservoir (242) und den das Reservoir verlassenden Kapillarkanal (254), wobei die Kontrolleinrichtung
eine Lichtquelle (246), die so angeordnet ist, daß sie auf das Fluid in dem Reservoir (242) einwirkt;
einen Lichtdetektor (252), der in enger Nähe zu dem Kapillarkanal (254) angeordnet ist, so daß er Licht, das durch die Teilchen in den Kapillarkanal und weiterhin von den Teilchen unter Austritt durch die Wände des Kapillarkanals (254) reflektiert wird, sammelt;
einen Signalgenerator (248), der mit der Lichtquelle (246) zusammenwirkt und mit dieser verbunden ist, wobei ein meßbares Signal auf die Emission der Lichtquelle gelegt wird; und
einen Filter (256), der mit der Emission des Lichtdetektors (252) verbunden ist und mit dieser zusammenwirkt, wobei das meßbare Signal von anderen Lichtquellen, die auf den Lichtdetektor (252) auftreffen können, isoliert ist,
umfaßt.

20. Kontrolleinrichtung nach Anspruch 19, bei der die Lichtquelle (246) infrarotes Licht liefert und das meßbare Signal eine periodische Variation in der Intensität des Lichtes ist.

21. Kontrolleinrichtung nach Anspruch 19, bei der Licht von der Lichtquelle (246) auf das Fluid in dem Reservoir (242) in einem rechten Winkel zu einer von dem Kapillarkanal (254) gebildeten Linie auftrifft.

22. Kontrolleinrichtung nach Anspruch 19, bei der die Lichtquelle (246) eine infrarote Lichtquelle ist, der Kapillarkanal (254) mit dem Reservoir (242) an einer Kapillaröffnung verbunden ist, die Lichtquelle (246) in einem Abstand von 0,5 bis 2 mm von dem Kapillareintritt angeordnet ist und der Lichtdetektor (252) in einem Abstand von 1 bis 4 mm von dem Kapillareintritt angeordnet ist.

23. Kontrolleinrichtung nach Anspruch 19, bei der das Licht von der Lichtquelle (246), das in das Probenreservoir (242) eintritt, in einem im wesentlichen rechten Winkel von der Lichtquelle in den Kapillarkanal (254) reflektiert wird und den Kapillarkanal (254) in im wesentlichen rechten Winkel zu Auftreffen auf den Detektor (252) verläßt.

24. Analysenvorrichtung zur Bestimmung der Anwesenheit eines Analyten in einer Probe einer physiologischen Flüssigkeit, enthaltend:
ein Gehäuse (162) mit einer Einlaßöffnung (170), einer Kammereinheit (176), einer Austrittsöffnung (180), einer ersten Kapillareinheit (168) für das unabhängige Pumpen einer Flüssigkeit von der Einlaßöffnung (180) zu der Kammereinheit (176), und einer zweiten Kapillareinheit (172), die zwischen der Kammereinheit (176) und der Austrittsöffnung (180) angeordnet und mit diesen wirksam verbunden

ist, zum unabhängigen Pumpen einer Flüssigkeit von der Kammereinheit (176) zu der Austrittsöffnung (180); wobei die Eintrittsöffnung (170), die erste Kapillareinheit (168), die Kammereinheit (176), die zweite Kapillareinheit (172) und die Austrittsöffnung (180) in einem fortlaufenden Kapillarkanal vorhanden sind, wobei das Gehäuse (162) weiterhin ein Reagenz in dem Kapillarkanal enthält, welches ein Mitglied aus der Gruppe der Verbindungen, die die Blutgerinnung bewirken, und der Antikörper, die eine Veränderung in der Strömungsgeschwindigkeit der Fluidprobe in dem Kanal bewirken, umfaßt.

**25.** Vorrichtung nach Anspruch 24, bei der das Reagenz einen Antikörper umfaßt.

**26.** Vorrichtung nach Anspruch 24, enthaltend mindestens zwei Kammereinheiten in dem Kanal.

**27.** Vorrichtung nach Anspruch 24, enthaltend eine verschließbare Belüftungsöffnung in dem Kapillarkanal proximal zu einer Verbindung zwischen einer Kammereinheit und einer Kapillareinheit.

**28.** Vorrichtung nach Anspruch 24, enthaltend eine dritte Kapillareinheit, die im wesentlichen parallel zu der ersten Kapillareinheit verläuft und Fluid in die Kammereinheit leitet.

**29.** Vorrichtung nach Anspruch 24, bei der das Reagenz ein Mitglied aus der Gruppe umfaßt, welche aus Komponenten der natürlichen Blutgerinnungskaskaden und eine Komponente einer natürlichen Gerinnungskaskade aktivierende Reagenzien gebildet ist.

**30.** Vorrichtung nach Anspruch 24 oder 29, bei der das Reagenz Thromboplastin ist.

**31.** Vorrichtung nach Anspruch 24, bei der das Gehäuse ein im wesentlichen hydrophobes Material umfaßt, wobei mindestens ein Bereich der Wende des Kapillarkanals zum Erhalt einer Hydrophilie behandelt ist.

**32.** Vorrichtung nach Anspruch 24 oder 31, bei der das Material ein Kunststoff ist.

**33.** Vorrichtung nach Anspruch 24, die aus mindestens zwei Bahnen eines im wesentlichen hydrophoben Kunststoffs hergestellt ist, wobei mindestens einer so geformt ist, daß er Vertiefungen für die Bildung der Kapillar- und Kammereinheiten liefert, wobei die Vertiefungen zur Schaffung der Hydrophilie behandelt sind und wobei mindestens eine Bahn eine eine Öffnung bildende Einrichtungen aufweist, die die Eintrittsöffnung oder die Austrittsöffnung bildet, und wobei durch das Verbinden der zwei Folien unter Erhalt des Gehäuses die Kapillar- und Kammereinheiten gebildet werden.

**34.** Vorrichtung nach Anspruch 24 oder 33, bei der die Kammereinheit das Reagenz enthält und das Reagenz ein Mitglied aus der Gruppe umfaßt, die aus Komponenten natürlicher Gerinnungskaskaden und eine Komponente einer natürlichen Gerinnungskaskade aktivierendem Reagenz gebildet ist.

**35.** Vorrichtung gemäß einem jeden der Ansprüche 24, 33 oder 34, bei der das Reagenz Thromboplastin in der Kammereinheit umfaßt.

**36.** Vorrichtung zur Verwendung in einem System zur Bestimmung der Anwesenheit eines Analyten in einer Blutprobe, enthaltend:
eine Vorrichtung wie in Anspruch 1 beschrieben, wobei der Kapillarkanal ein im wesentlichen hydrophobes Gehäuse umfaßt, das mindestens einen Wandbereich aufweist, der zur Bildung einer Hydrophilie behandelt ist, und wobei der Kapillarkanal Blut als die einzige beweglichen Quelle in der Vorrichtung pumpen kann, und
ein Reagenz in dem Kanal, wobei das Reagenz die Gerinnung von Blut verursacht und ein Mitglied eines Systems ist, das ein meßbares Signal bezüglich der Anwesenheit des Analyten bzw. der Eigenschaft liefern kann.

**37.** Vorrichtung gemäß einem jeden der Ansprüche 24 bis 36, dadurch gekennzeichnet, daß das Reagenz an den Wänden des Kapillarkanals befestigt ist.

## Revendications

1. Méthode pour déterminer la présence d'une quantité d'un produit à analyser dans un échantillon de fluide, ou une propriété de cet échantillon de fluide, qui consiste à :

appliquer ledit échantillon à un dispositif (10) comprenant un orifice d'entrée (14) pour ledit échantillon, une lumière (22), un parcours capillaire contenant une chambre (12, 20) reliant ledit orifice d'entrée (14) à ladite lumière (22) et un réactif (16, 24) dans ledit parcours capillaire (12, 20), méthode dans laquelle ledit échantillon s'écoule par ledit parcours (12, 20) sous l'action des forces capillaires, une interaction dudit réactif (16, 24) avec ledit échantillon modifiant la viscosité dudit échantillon ou une caractéristique dudit échantillon associée audit écoulement ;

à permettre audit échantillon d' interagir avec ledit réactif (16, 24) et de traverser au moins une partie dudit parcours capillaire (12, 20) ;

à détecter ladite viscosité ou caractéristique d'écoulement ; et

à relier ladite viscosité ou caractéristique d'écoulement à la présence ou à la quantité dudit produit à analyser dans ledit échantillon de fluide, ou à ladite propriété dudit échantillon .

2. Méthode selon la revendication 1, dans laquelle , soit ledit échantillon, soit ledit réactif (16,24) comprend des particules , ladite caractéristique d'écoulement implique un passage de ces particules à travers ledit parcours capillaire (12,20).

3. Méthode selon la revendication 1 ou 2, dans laquelle ladite caractéristique d'écoulement comprend une modification de la distribution en taille des particules.

4. Méthode selon l'une quelconque des revendications 1 à 3, dans laquelle ladite modification dans la distribution de la taille des particules provient de l'agglutination de ces particules.

5. Méthode selon l'une quelconque des revendications 1 à 4, dans laquelle les globules rouges d'un échantillon de sang entier fonctionnent comme des particules dans ledit parcours capillaire (12, 20) et ladite caractéristique d'écoulement comprenant l'interruption d'un faisceau lumineux dirigé à travers ledit parcours capillaire (12, 20) par lesdits globules rouges, lorsque ces globules passent à travers le parcours (12, 20).

6. Méthode selon l'une quelconque des revendications 1 à 5, dans laquelle des particules sont présentes dans ledit échantillon.

7. Méthode selon l'une quelconque des revendications 1 à 5, dans laquelle des particules sont présentes dans ledit réactif (16, 24).

8. Méthode selon l'une quelconque des revendications 1 à 7, dans laquelle ladite caractéristique d'écoulement comprend une modification de la vitesse de passage des particules dans ledit parcours (12,20).

9. Méthode selon l'une quelconque des revendications 1 à 7, dans laquelle ladite caractéristique d'écoulement est une modification de la vitesse d'écoulement de l'échantillon à travers ledit parcours capillaire (12, 20).

10. Méthode selon l'une quelconque des revendications 1 à 9, dans laquelle ledit échantillon est constitué de sang entier.

11. Méthode selon l'une quelconque des revendications 1 à 10, dans laquelle ladite modification d'écoulement consiste en un ralentissement ou un arrêt de l'écoulement, suite à la formation d'un caillot.

12. Méthode selon l'une quelconque des revendications 1 à 10, dans laquelle ladite modification d'écoulement est le démarrage de l'écoulement suite à la dissolution d'un caillot.

13. Méthode selon la revendication 1, dans laquelle ledit échantillon comprend du sang entier, ledit réactif (16,24) initiant une coagulation dudit échantillon.

14. Méthode selon la revendication 1, dans laquelle ledit parcours capillaire (12, 20) comprend au moins une chambre (20) contenant ledit réactif (24) et au moins une unité capillaire (12), ayant une surface de section transversale inférieure à la surface de section transversale de la chambre (20).

15. Méthode selon la revendication 14, dans laquelle ledit parcours capillaire comprend ledit orifice d'entrée, une unité de chambre, une première unité capillaire reliant ledit orifice d'entrée à ladite unité de chambre, une lumière, et une seconde unité capillaire reliant ladite unité de chambre à ladite lumière, dans laquelle un échantillon appliqué audit orifice d'entrée est transporté par ledit premier capillaire à ladite unité de chambre et est transporté par ladite seconde unité capillaire de ladite unité unité de chambre vers ladite lumière.

16. Méthode selon la revendication 15, dans laquelle ledit dispositif comporte un troisième orifice, proche d'une liaison de ladite chambre avec ladite seconde unité capillaire, dans laquelle la transmission dudit échantillon s'achève au niveau du troisième orifice pour permettre une incubation dudit échantillon avec ledit réactif, ladite méthode comprenant , de plus, une étape de fermeture étanche dudit troisième orifice après ladite incubation pour permettre un mouvement continu dudit échantillon de ladite unité de chambre vers ladite seconde unité capillaire.

17. Méthode selon la revendication 1, dans laquelle plus d'un parcours capillaire reçoit un échantillon issu d'un seul orifice d'entrée.

18. Méthode selon la revendication 17, dans laquelle chacun desdits parcours capillaires contient un réactif différent.

19. Dispositif de contrôle capable de détecter la déplétion d'un fluide contenant des particules dans un dispositif selon la revendication 1 contenant un réservoir d'échantillon (242), ledit parcours capillaire (254) sortant dudit réservoir (242), lequel comprend :

une source lumineuse (246), disposée de façon à être captée par ledit fluide dans ledit réservoir (242) ;

un détecteur de lumière (252), disposé tout près dudit parcours capillaire (254) de façon à recueillir la lumière qui est réfléchie par lesdites particules dans ledit parcours capillaire (254) et ensuite réfléchie ,de plus, par lesdites particules, de façon à traverser les parois dudit parcours capillaire (254) ;

un générateur de signaux (248), fixé de façon opérationnelle à ladite source lumineuse (246), dans lequel un signal détectable est imposé à la sortie de ladite source lumineuse ; et

un filtre (256), fixé de façon opérationnelle à la sortie dudit détecteur de lumière (252) , dans lequel ledit signal détectable est isolé des autres sources lumineuses qui peuvent être captées par ledit détecteur de lumière (252).

20. Dispositif de contrôle selon la revendication 19, dans lequel ladite source lumineuse (246) produit une lumière infrarouge et ledit signal détectable est une variation périodique de l'intensité de ladite lumière .

21. Dispositif de contrôle selon la revendication 19, dans lequel une lumière issue de ladite source lumineuse (246) rencontre ledit fluide dans ledit réservoir (242), perpendiculairement à la ligne formée par ledit parcours capillaire (254).

22. Dispositif de contrôle selon la revendication 19, dans lequel ladite source lumineuse (246) est une source de lumière infrarouge , ledit parcours capillaire (254) étant relié audit réservoir (242) au niveau de l'entrée du capillaire, ladite source lumineuse (246) étant située à une distance de 0,5 à 2mm de ladite entrée du capillaire, et ledit détecteur de lumière (252) étant placé à distance de 1 à 4 mm de ladite entrée du capillaire.

23. Dispositif de contrôle selon la revendication 19, dans lequel une lumière issue de ladite source lumineuse (246) pénétrant dans ledit réservoir d'échantillon (242), est réfléchie essentiellement perpendiculairement à partir de ladite source lumineuse (246) dans ledit parcours capillaire (254) et sort dudit parcours capillaire (254) essentiellement perpendiculairement, pour être captée par ledit détecteur (252).

24. Dispositif d'analyse pour détecter la présence d'un produit à analyser dans un échantillon de fluide physiologique , comprenant :

une enceinte (162), contenant un orifice d'admission (170), une unité de chambre (176), un orifice de sortie (180), une première unité capillaire (168) pour pomper de façon indépendante un liquide venant dudit orifice d'admission (170) vers l'unité de chambre (176), et une seconde unité capillaire (172) positionnée entre et raccordée directement à ladite unité de chambre (176) et audit orifice de sortie (180) pour pomper de façon indépendante un liquide venant de ladite unité de chambre (176) vers ledit orifice de sortie (180) ; dans lequel ledit orifice d'admission (170), la première unité capillaire (168), l'unité de chambre (176), la seconde unité capillaire (172), l'orifice de sortie (180) sont situés sur un parcours capillaire continu ; ladite enceinte (162) contenant de plus un réactif dans ledit parcours capillaire comprenant un élément choisi dans le groupe constitué de composés provoquant une coagulation du sang et des anticorps capables d'entrainer une modification dans la vitesse de l'écoulement dudit échantillon de fluide dans ledit parcours.

25. Dispositif selon la revendication 24, dans lequel ledit réactif comprend un anticorps.

26. Dispositif selon la revendication 24, dans lequel ledit dispositif comprend au moins deux unités de chambre dans ledit parcours.

27. Dispositif selon la revendication 24, comprenant de plus un orifice pouvant être étanche dans ledit parcours capillaire, proche d'une jonction entre l'unité de chambre et une unité capillaire.

28. Dispositif selon la revendication 24, comprenant de plus, une troisième unité capillaire, pratiquement en parallèle avec ladite première unité et dirigeant le fluide dans ladite unité de chambre.

29. Dispositif selon la revendication 24, dans lequel ledit réactif comprend un élément choisi dans un groupe consistant en composants de cascades de coagulations sanguines naturelles et de réactifs activant un composant d'une cascade de coagulation naturelle.

30. Dispositif selon la revendication 24 ou 29, dans lequel ledit réactif est la thromboplastine.

31. Dispositif selon la revendication 24, dans lequel ladite enceinte comprend un matériau pratiquement hydrophobe, ayant au moins une partie des parois dudit parcours capillaire traitée pour fournir une capacité hydrophile.

32. Dispositif selon la revendication 24 ou 31, dans lequel ledit matériau est un plastique.

33. Dispositif selon la revendication 24, fabriqué à partir d'au moins deux plaques de plastique pratiquement hydrophobe, dont l'une au moins est moulée pour fournir des dépressions pour la formation desdites unités de chambre et des capillaires, lesdites dépressions étant traitées pour présenter une capacité hydrophile, dans lequel au moins une plaque comporte des moyens définissant un passage formant ledit orifice d'admission ou ledit orifice de sortie, lesdites unités de chambre et les capillaires étant formés par la jonction desdites plaques pour constituer ladite enceinte.

34. Dispositif selon la revendication 24 ou 33, dans lequel ladite unité de chambre contient ledit réactif et en ce que ledit réactif comprend un élément choisi dans le groupe constitué de composants des cascades de coagulations naturelles et de réactifs activant un composant d'une cascade de coagulation naturelle.

35. Dispositif selon l'une quelconque des revendications 24, 33 ou 34, dans lequel ledit réactif comprend de la thromboplastine, dans ladite unité de chambre.

36. Dispositif à utiliser dans un système de détection de la présence d'un produit à analyser dans un échantillon sanguin, comprenant :

un dispositif selon la revendication 1, dans lequel ledit parcours capillaire comprend une enceinte pratiquement hydrophobe, comportant une partie au moins de ses parois traitée pour avoir des capacités hydrophiles, et étant capable de pomper du sang comme unique source mobile dudit dispositif ; et

un réactif dans ledit parcours, dans lequel ledit réactif entraine le sang à coaguler et est un élément d'un système capable de fournir un signal détectable en relation avec la présence dudit produit à analyser ou de ladite propriété .

37. Dispositif selon l'une quelconque des revendications 24 à 36, caractérisé en ce que le réactif est fixé aux parois dudit parcours capillaire.

FIG. 1A

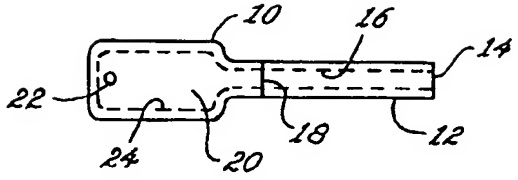



FIG. 1B

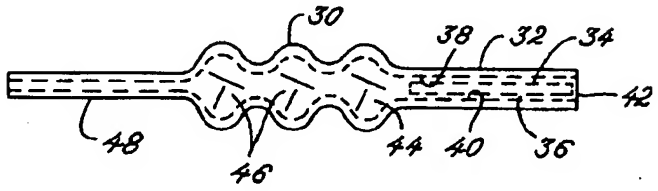



FIG. 2A

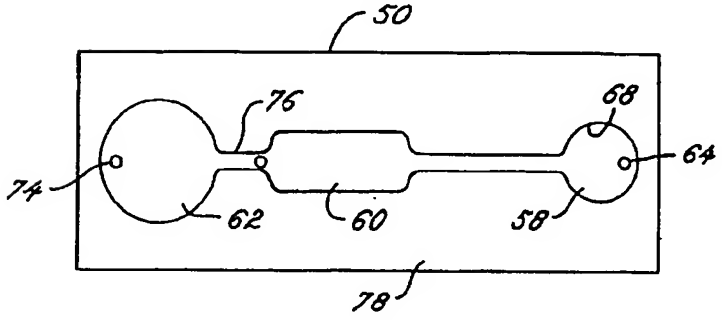



FIG. 2B

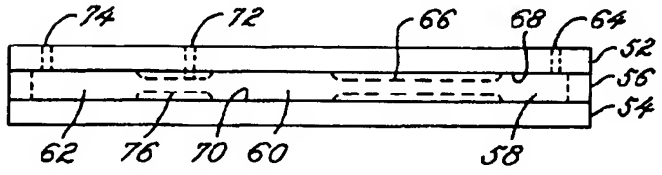


FIG. 3

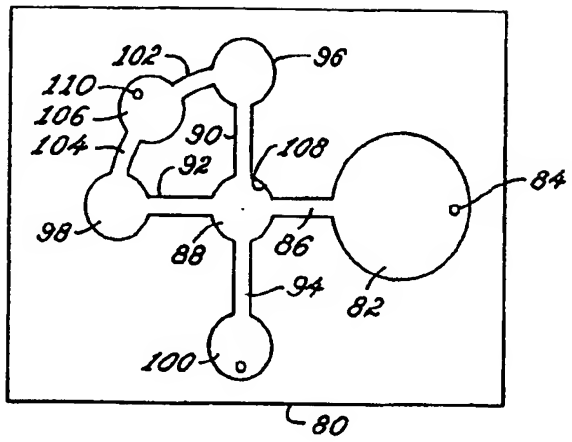



FIG. 4

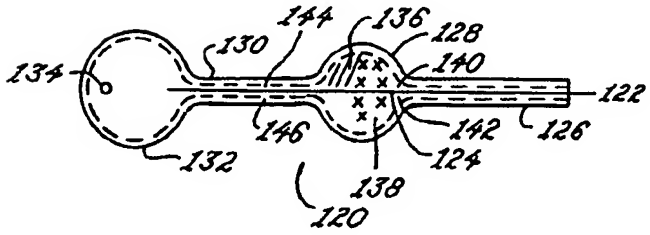



FIG. 5

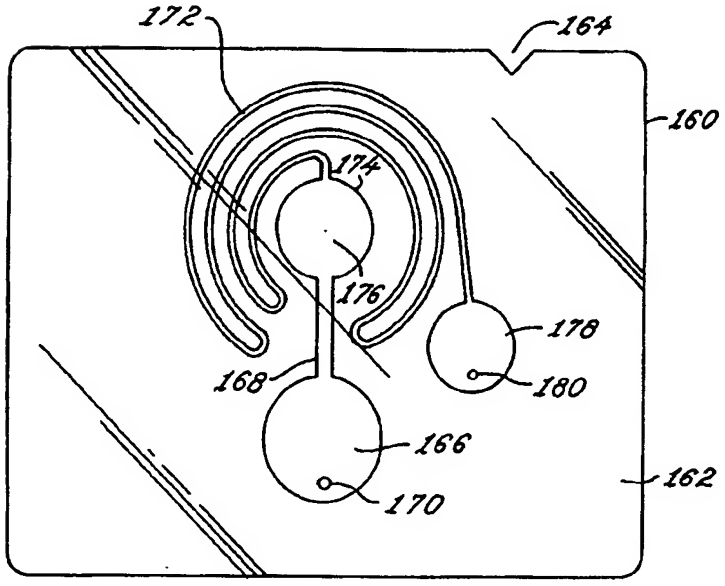


FIG. 6A

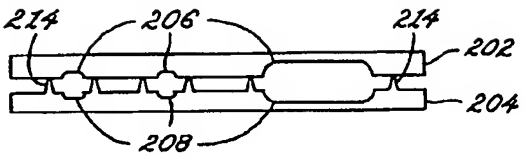



FIG. 6B

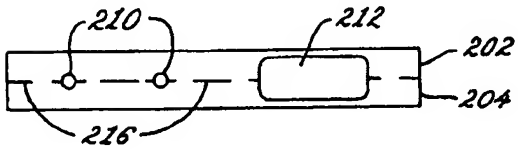



FIG. 7

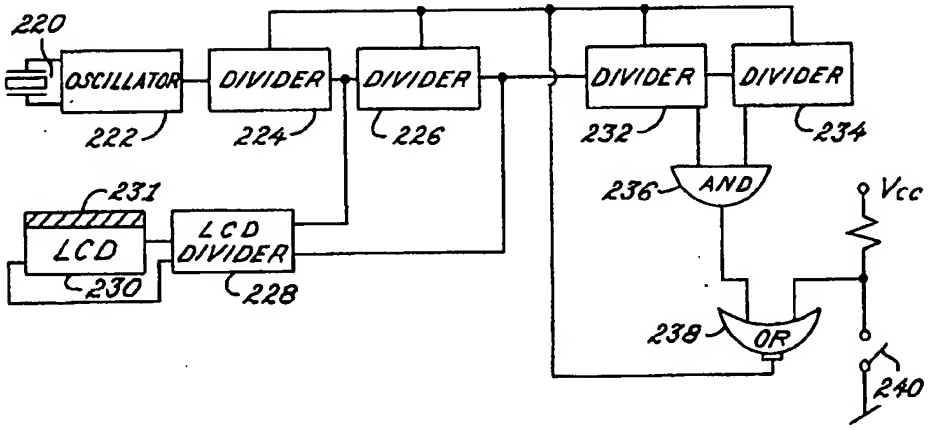
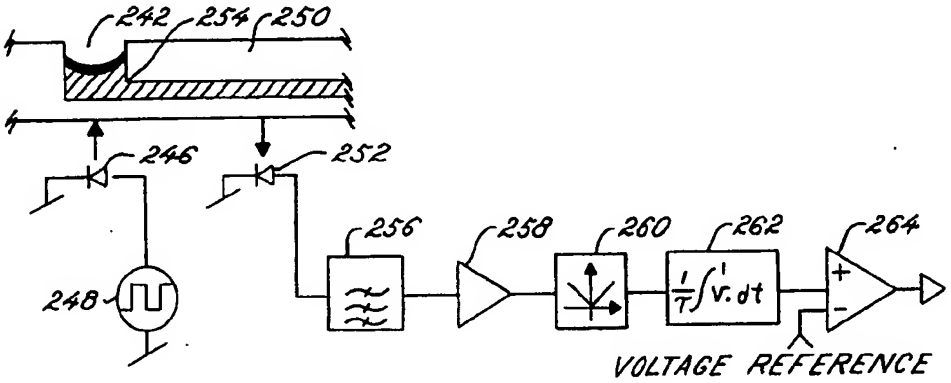



FIG. 8



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(54) **Rapid detection of nucleic acid sequences in a sample by labeling the sample.**

(57) A method for detecting one or more microorganisms or polynucleotide sequences from eukaroytic sources in a nucleic acid-containing test sample comprising

(a) preparing a test sample comprising labeling the nucleic acids in the test sample,

(b) preparing one or more process by immobilizing a single-stranded nucleic acid of one or more known microorganisms or sequences from eukaroytic sources,

(c) contacting, under hybridization conditions, the labeled single-stranded nucleic acid to form hybridized labeled nucleic acids, and

(d) assaying for the hybridized nucleic acids by detecting the label. The method can be used to detect genetic disorders, e.g., sickle-cell anemia.

EP 0 235 726 A2

# RAPID DETECTION OF NUCLEIC ACID SEQUENCES IN A SAMPLE BY LABELING THE SAMPLE

## BACKGROUND OF THE INVENTION

### Field of the Invention

The present application relates to the detection and identification of microorganisms and the detection and identification of particular prokaryotic or eukaryotic DNA sources in a nucleic acid containing test sample.

Still further, the present invention relates to a method for the lysis of whole cells.

### Background Information

#### A. The Detection of Microorganisms

The identification of species of microorganisms in a sample containing a mixture of microorganisms, by immobilizing the DNA from the sample and subjecting it to hybridization with a labelled specimen of species -specific DNA from a known microorganism and observing whether hybridization occurs between the immobilized DNA and the labelled specimen, has been disclosed in PCT patent application No. PCT/US83/01029.

The most efficient and sensitive method of detection of nucleic acids such as DNA after hybridization requires radioactively labeled DNA. The use of autoradiography and enzymes prolongs the assay time and requires experienced technicians.

U.S.P. 4,358,535 to Falkow et al describe infectious disease diagnosis using labeled nucleotide probes complementary to nucleic acid coding for a characteristic pathogen product.

#### B. The Detection of Specific Eukaryotic Sequences

The identification of specific sequence alteration in an eukaryotic nucleic acid sample by immobilizing the DNA from the sample and subjecting it to hybridization with a labeled oligonucleotide and observing whether hybridization occurs between the immobilized DNA and the labeled probe, has been described in EP -patent application No. 86 117 978 filed December 23, 1986, now pending.

It is known that the expression of a specific gene determines the physical condition of a human being. For example, a change in the beta-globin gene coding sequence from GAG to GTG at the sixth amino acid position produces sickle-beta-globin and a homozygote can have a disease known as sickle cell anemia. Similarly deletion of particular sequences from alpha-globin or beta-globin genes can cause thalassemias. A recent survey, The New Genetics and Clinical Practice, D.J. Weatherall, The Nuffield Provincial Hospitals Trust, (1982), chapter 2 describes the frequency and clinical spectrum of genetic diseases.

Problems associated with genetic defects can be diagnosed by nucleic acid sequence information. The easiest way to detect such sequence information is to use the method of hybridization with a specific probe of a known sequence.

U.S.P. 4,395,486 to Wilson et al describe a method for the direct analysis of sickle cell anemia using a restriction endonuclease assay.

Edward M. Rubin and Yuet Wai Kan, "A Simple Sensitive Prenatal Test for Hydrops Fetalis Caused By α-Thalassaemia", The Lancet, January 12, 1985, pp. 75-77 describes a dot blot analysis to differentiate between the genotypes of homozygous alpha-thalassemia and those of the haemoglobin-H disease and alpha-thalassemia trait.

The most efficient and sensitive method of detection of nucleic acids, such as DNA, after hybridization requires radioactively labelled DNA. The use of autoradiography and enzymes prolongs the assay time and requires experienced technicians.

Recently, a non-radioactive method of labelling DNA was described by Ward et al, European Patent Application 63,879. Ward et al, use the method of nick translation to introduce biotinylated U (uracil) residues into DNA, replacing T (thymine). The biotin residue is then assayed with antibiotin antibody or an avidin-containing system. The detection in this case is quicker than autoradiography, but the nick translation

method requires highly skilled personnel. Moreover, biotinylation using biotinylated UTP (uridine triphosphate) works only for thymine-containing polynucleotides. The use of other nucleoside triphosphates is very difficult because the chemical derivatization of A (adenine) or G (guanine) or C (cytosine) (containing $-NH_2$) with biotin requires the skills of trained organic chemists.

C. Cell Lysis

The present invention also provides a method for the efficient lysis of whole cells such that their DNA is released and made available for photochemical labeling. While eukaryotic cells derived from multicellular animals are easily lysed under relatively mild conditions, single cell eukaryotes and prokaryotes, especially Gram positive prokaryotes, are more difficult to lyse due to the complicated chemical nature and extent of cross-linking of their cell walls. Methods do exist for efficiently lysing these refractory organisms, either by chemical-enzymatic or physical means, but these methods are often complicated, time-consuming and inappropriate for preserving the integrity of DNA.

## SUMMARY OF THE INVENTION

It is accordingly an object of the present invention to provide a method for detection of microorganisms in a nucleic acid-containing test sample.

It is another object of the invention to provide a method for a simultaneous assay for the presence of more than one nucleic acid sequence.

Another object is to provide a method to identify particular prokaryotic or eukaryotic DNA sequences and a method for distinguishing alleles of individual genes.

Another object of the invention is to provide a simple photochemical method of labeling the unknown test sample.

A further object of the invention is to label the probes with different kinds of labels so that when the probes are hybridized with an immobilized, unknown, unlabelled test sample, the type of label remaining bound after hybridization and washing, will determine the type of nucleic acid sequence present in the unknown sample.

A still further object of the invention is to use whole chromosomal nucleic acid as the probe and/or as the test sample.

Also the invention relates to the use of oligonucleotides as immobilized probes.

These and other objects and advantages are realized in accordance with the present invention for a method of detecting nucleic acid sequences in a nucleic acid-containing test sample.

The method involves the following:

(a) preparing a test sample comprising labeling the nucleic acids of the organisms or cells or cell debris in the test sample,

(b) preparing one or more probes by immobilizing a single-stranded DNA or an oligonucleotide of one or more known microorganisms or eukaryotes, or sequences representing particular genes or their alleles,

(c) contacting, under hybridization conditions, the labeled single-stranded sample nucleic acid and the immobilized single-stranded (probe) nucleic acid or the immobilized oligonucleotide to form hybridized labeled nucleic acids and

(d) assaying for the hybridized nucleic acids by detecting the label.

In the above method, steps (a) and (b) can be reversed.

The method further comprises denaturing the labeled nucleic acids from step (a) to form labeled denatured nucleic acids.

According to the invention, a labeled nucleic acid test sample is contacted simultaneously with several different types of DNA probes for hybridization. The nucleic acid test sample is labeled and hybridized with several unlabeled immobilized probes. The positions of the probes are fixed, and the labeled probe detected after hybridization will indicate that the test sample carries a nucleic acid sequence complementary to the corresponding probe.

Nucleic acid probes for several microbiological systems or for different alleles of one or more genes can be immobilized separately on a solid support, for example, nitrocellulose paper. The test sample nucleic acids are labeled and remain in solution. The solid material containing the immobilized probe is brought in contact with the labeled test nucleic acid solution under hybridization conditions. The solid

material is washed free of unhybridized nucleic acid and the label is assayed. The presence of the label with one or more of the probes indicates that the test sample contains nucleic acids substantially complementary to those probes and hence originate, for example, from an infection by particular microbiological systems.

Labeling can be accomplished in a whole living cell or a cell lysate, and can be non-isotopic. The nucleic acid can be used for hybridization without further purification.

The present invention also concerns specific lysis conditions to release nucleic acids from both gram positive and gram negative bacteria.

The present invention further concerns a kit for detecting microorganisms or eukaryotes in a test sample comprising

(a) a support solid containing single-stranded DNA of one or more known microorganisms or eukaryotes immobilized thereon, e.g., a strip containing dots or spots of known microorganisms or eukaryotes,

(b) a reagent for labeling the nucleic acid of the test sample,

(c) a reagent for releasing and denaturing DNA in the test sample, and

(d) hybridization reagents.

For chemiluminescence detection of the hybridized nucleic acid, the kit would further comprise a reagent for chemiluminescent detection.

In the above described kit, the reagent for labeling is given hereinbelow in a discussion on labels.

Reagents for releasing and denaturing DNA include sodium hydroxide and lysing agents such as detergents and lysozymes.

Typical hybridization reagents includes a micture of sodium chloride, sodium citrate, SDS (sodium dodecyl sulfate), bovine serum albumin, nonfat milk or dextran sulfate and optionally formamide.

## BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is an autoradiograph of results of immobilization of an oligonucleotide sequence specific for hemoglobin mutation.

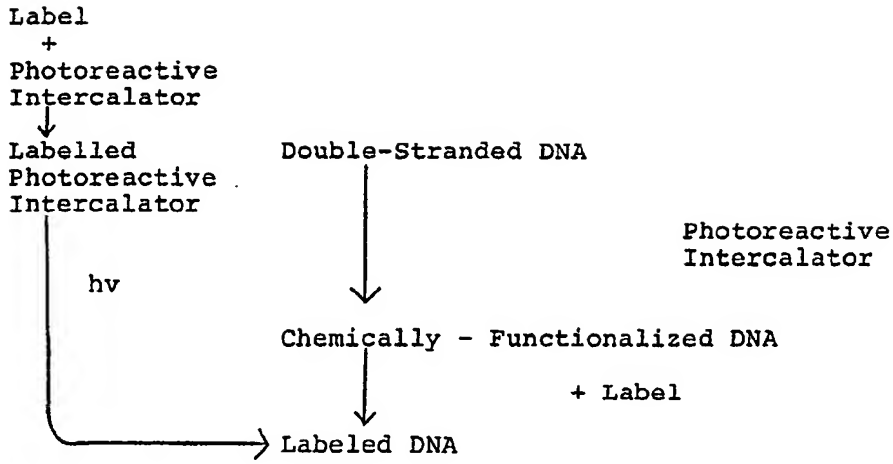
Fig. 2 is a photograph of results of hybridization with labeled genomic DNA for non radioactive detection.

## DETAILED DESCRIPTION OF THE INVENTION

The nucleic acid is preferably labeled by means of photochemistry, employing a photoreactive DNA-binding furocoumarin or a phenanthridine compound to link the nucleic acid to a label which can be "read" or assayed in conventional manner, including fluorescence detection. The end product is thus a labeled nucleic acid comprising (a) a nucleic acid component, (b) an intercalator or other DNA-binding ligand photochemically linked to the nucleic acid component, and (c) a label chemically linked to (b).

The photochemical method provides more favorable reaction conditions than the usual chemical coupling method for biochemically sensitive substances. The intercalator and label can first be coupled and then photoreacted with the nucleic acid, or the nucleic acid can first be photoreacted with the intercalator and then coupled to the label.

A general scheme for coupling a nucleic acid, exemplified by double-stranded DNA, to apply a label, is as follows:




Where the hybridizable portion of the nucleic acid is in a double stranded form, such portion is then denatured to yield a hybridizable single stranded portion. Alternatively, where the labeled DNA comprises the hybridizable portion already in single stranded form, such denaturation can be avoided if desired. Alternatively, double stranded DNA can be labeled by the approach of the present invention after hybridization has occurred using a hybridization format which generates double stranded DNA only in the presence of the sequence to be detected.

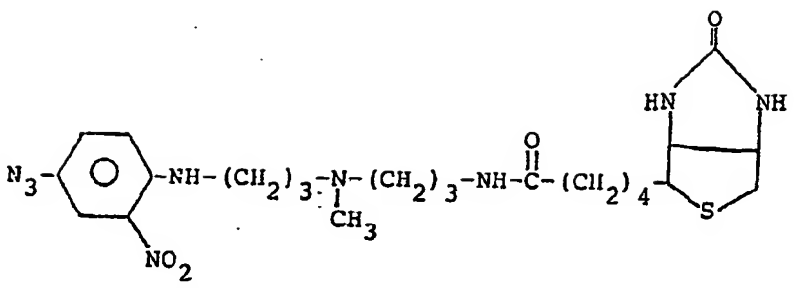
To produce specific and efficient photochemical products, it is desirable that the nucleic acid component and the photoreactive intercalator compound be allowed to react in the dark in a specific manner.

For coupling to DNA, aminomethyl psoralen, aminomethyl angelicin and amino alkyl ethidium or methidium azides are particularly useful compounds. They bind to double-stranded DNA and only the complex produces photoadduct. In the case where labeled double-stranded DNA must be denatured in order to yield a hybridizable single stranded region, conditions are employed so that simultaneous interaction of two strands of DNA with a single photoadduct is prevented. It is necessary that the frequency of modification along a hybridizable single stranded portion of the nucleic acid not be so great as to substantially prevent hybridization, and accordingly there preferably will be not more than one site of modification per 25, more usually 50, and preferably 100, nucleotide bases. Angelicin derivatives are superior to psoralen compounds for monoadduct formation. If a single-stranded DNA nucleic acid is covalently attached to some extra double-stranded DNA, use of phenanthridium and psoralen compounds is desirable since these compounds interact specifically to double-stranded DNA in the dark. The chemistry for the synthesis of the coupled reagents to modify nucleic acids for labeling, described more fully hereinbelow, is similar for all cases.

The nucleic acid component can be single or double stranded DNA or RNA or fragments thereof such as are produced by restriction enzymes or even relatively short oligomers.

The DNA-binding ligands of the present invention used to link the nucleic acid component to the label can be any suitable photoreactive form of known DNA-binding ligands. Particularly preferred DNA-binding ligands are intercalator compounds such as the furocoumarins, e.g., angelicin (isopsoralen) or psoralen or derivatives thereof which photochemically will react with nucleic acids, e.g., 4'-aminomethyl-4,5'-dimethyl angelicin, 4'-aminomethyl-trioxsalen (4'aminomethyl-4,5',8-trimethyl-psoralen), 3-carboxy-5-or -8-amino-or-hydroxy-psoralen, as well as mono-or bis-azido aminoalkyl methidium or ethidium compounds.

Particularly useful photoreactive forms of intercalating agents are the azidointercalators. Their reactive nitrenes are readily generated at long wavelength ultraviolet or visible light and the nitrenes of arylazides prefer insertion reactions over their rearrangement products (see White et al, Methods in Enzymol., 46, 644 (1977)). Representative intercalating agents include azidoacridine, ethidium monoazide, ethidium diazide, ethidium dimer azide (Mitchell et al, JACS, 104, 4265 (1982)), 4-azido-7-chloroquinoline, and 2-azidofluorene. A specific nucleic acid binding azido compound has been described by Forster et al, Nucleic Acid Res., 13, (1985), 745. The structure of such compound is as follows:



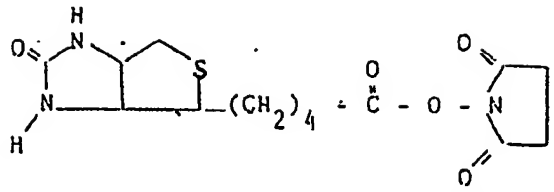
Other useful photoreactable intercalators are the furocoumarins which form (2+2) cycloadducts with pyrimidine residues. Alkylating agents can also be used such as bis-chloroethylamines and epoxides or aziridines, e.g., aflatoxins, polycyclic hydrocarbon epoxides, mitomycin and norphillin A.

Nonlimiting examples of intercalator compounds for use in the present invention include acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines and quinolines.

The label which is linked to the nucleic acid component according to the present invention can be any chemical group or residue having a detectable physical or chemical property, i.e., labeling can be conducted by chemical reaction or physical adsorption. The label will bear a functional chemical group to enable it to be chemically linked to the intercalator compound. Such labeling materials have been well developed in the field of immunoassays and in general most any label useful in such methods can be applied to the present invention. Particularly useful are enzymatically active groups, such as enzymes (see Clin. Chem., (1976), 22, 1243), enzyme substrates (see British Pat. Spec. 1,548,741), coenzymes (see U.S. Patent Nos. 4,230,797 and 4,238,565) and enzyme inhibitors (see U.S. Patent No. 4,134,792; fluorescers - (see Clin. Chem. , (1979), 25, 353), and chromophores including phycobiliproteins; luminescers such as chemiluminescers and bioluminescers (see Clin. Chem., (1979), 25, 512, and ibid, 1531); specifically bindable ligands, i.e., protein binding ligands; and residues comprising radioisotopes such as $^{3}H$, $^{35}S$, $^{32}P$, $^{125}I$, and $^{14}C$. Such labels are detected on the basis of their own physical properties (e.g., fluorescers, chromophores and radioisotopes) or their reactive or binding properties (e.g., enzymes, substrates, coenzymes and inhibitors). For example, a cofactor-labeled nucleic acid can be detected by adding the enzyme for which the label is a cofactor and a substrate for the enzyme. A hapten or ligand (e.g., biotin) labeled nucleic acid can be detected by adding an antibody or an antibody pigment to the hapten or a protein (e.g., avidin) which binds the ligand, tagged with a detectable molecule. An antigen can also be used as a label. Such detectable molecule can be some molecule with a measurable physical property - (e.g., fluorescence or absorbance) or a participant in an enzyme reaction (e.g., see above list). For example, one can use an enzyme which acts upon a substrate to generate a product with measurable physical property. Examples of the latter include, but are not limited to, beta-galactosidase, alkaline phosphatase, papain and peroxidase. For In situ hybridization studies, ideally the final product is water insoluble. Other labels, e.g., dyes, will be evident to one having ordinary skill in the art.

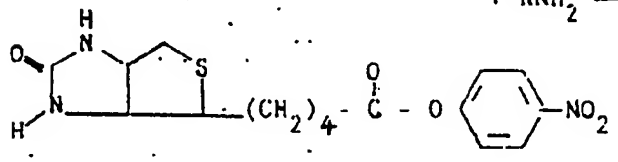
The label will be linked to the intercalator compound, e.g., acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines and quinolines, by direct chemical linkage such as involving covalent bonds, or by indirect linkage such as by the incorporation of the label in a microcapsule or liposome which in turn is linked to the intercalator compound. Methods by which the label is linked to the intercalator compounds are essentially known in the art and any convenient method can be used to perform the present invention.

Advantageously, the intercalator compound is first combined with label chemically and thereafter combined with the nucleic acid component. For example, since biotin carries a carboxyl group, it can be combined with a furocoumarin by way of amide or ester formation without interfering with the photochemical reactivity of the furocoumarin or the biological activity of the biotin, e.g.,

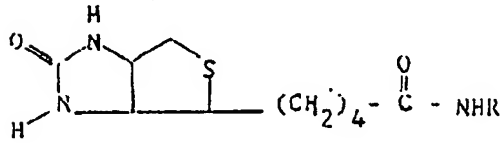
(i) 

Biotin-N-hydroxysuccinimide

or

(ii) 

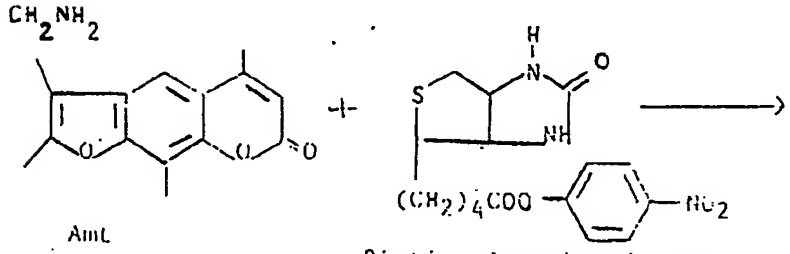
Biotin-p-nitrophenyl ester

(iii) 

or

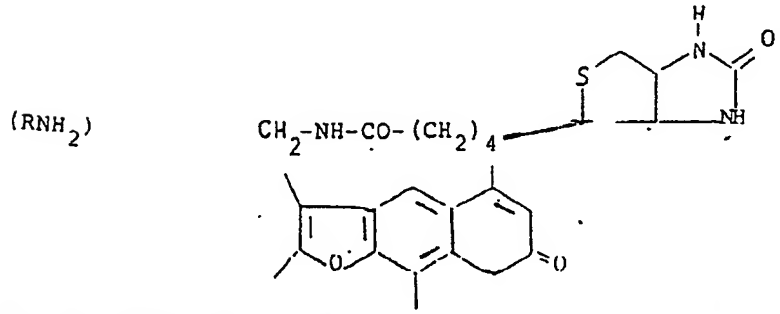
Biotin + ROH $\xrightarrow{\text{carbodiimide}}$ Biotin CO OR

By way of example,

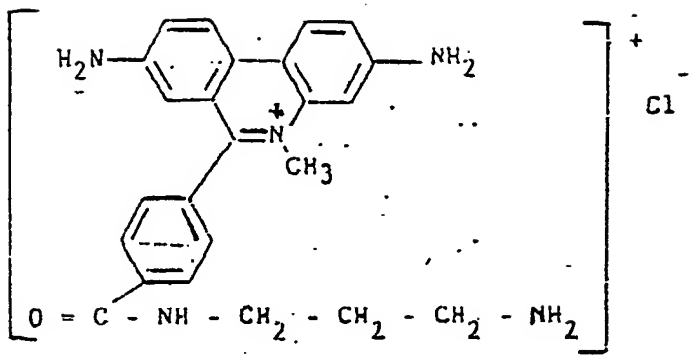


Amt

Biotin nitrophenyl ester



Other aminomethylangelicin, psoralen and phenanthridium derivatives can be similarly reacted, as can phenanthridium halides and derivatives thereof such as aminopropyl methidium chloride, i.e.,



(see Hertzberg et al, J. Amer. Chem. Soc., 104, 313 (1982)).

Alternatively, a bifunctional reagent such as dithiobis succinimidyl propionate or 1,4-butanediol diglycidyl ether can be used directly to couple the photochemically reactive molecule with the label where the reactants have alkyl amino residues, again in a known manner with regard to solvents, proportions and reaction conditions. Certain bifunctional reagents, possibly glutraldehyde may not be suitable because, while they couple, they may modify nucleic acid and thus interfere with the assay. Routine precautions can be taken to prevent such difficulties.

The particular sequence used in making the labeled nucleic acid can be varied. Thus, for example, an amino-substituted psoralen can first be photochemically coupled with a nucleic acid, the product having pendant amino groups by which it can be coupled to the label, i.e., labeling can be carried out by photochemically reacting a DNA binding ligand with the nucleic acid in the test sample. Alternatively, the psoralen can first be coupled to a label such as an enzyme and then to the nucleic acid.

As described in pending EP-patent application No. 85 116 199.2, filed December 18, 1985, the present invention also encompasses a labeled nucleic acid comprising (a) a nucleic acid component, (b) a nucleic acid-binding ligand photochemically linked to the nucleic acid component, (c) a label and (d) a spacer chemically linking (b) and (c).

Advantageously, the spacer includes a chain of up to about 40 atoms, preferably about 2 to 20 atoms, selected from the group consisting of carbon, oxygen, nitrogen and sulfur.

Such spacer may be the polyfunctional radical of a member selected from the group consisting of peptide, hydrocarbon, polyalcohol, polyether, polyamine, polyimine and carbohydrate, e.g., -glycyl-glycyl-glycyl-or other oligopeptide, carbonyl dipeptides, and omega-amino-alkane-carbonyl radical such as -NH-$(CH_2)_5$-CO-, a spermine or spermidine radical, an alpha, omega-alkanediamine radical such as -NH-$(CH_2)_6$-NH or -HN-$CH_2$-$CH_2$-NH, or the like. Sugar, polyethylene oxide radicals, glyceryl, pentaerythritol, and like radicals can also serve as the spacers.

These spacers can be directly linked to the nucleic acid-binding ligand and/or the label or the linkages may include a divalent radical of a coupler such as dithiobis succinimidyl propionate, 1,4-butanediol diglycidyl ether, a diisocyanate, carbodiimide, glyoxal, glutaraldehyde, or the like.

The spacer can be incorporated at any stage of the process of making the probe.

a-b-d-c

defined hereinabove. Thus, the sequence can be any of the following:

a+b+d+c,

b+d+c+a,

d+c+b+a,

b+d+a+c, etc.

The conditions for the individual steps are well known in chemistry.

If the label is an enzyme, for example, the product will ultimately be placed on a suitable medium and the extent of catalysis will be determined. Thus, if the enzyme is a phosphatase, the medium could contain nitrophenyl phosphate and one would monitor the amount of nitrophenol generated by observing the color. If the enzyme is a beta-galactosidase, the medium can contain o-nitro-phenyl-D-galacto-pyranoside which also will liberate nitrophenol.

The labeled nucleic acid of the present invention is applicable to all conventional hybridization assay formats, and in general to any format that is possible based on formation of a hybridization product or aggregate comprising the labelled nucleic acid. In particular, the unique labelled nucleic acid of the present invention can be used in solution and solid-phase hybridization formats, including, in the latter case, formats involving immobilization of either sample or probe nucleic acids and sandwich formats.

The nucleic acid probe will comprise at least one single-stranded base sequence substantially complementary to or homologous with the sequence to be detected. However, such base sequence need not be a single continuous polynucleotide segment, but can be comprised of two or more individual segments interrupted by nonhomologous sequences. These nonhomologous sequences can be linear or they can be self-complementary and form hairpin loops. In addition, the homologous region of the probe can be flanked at the 3' -and 5' termini by nonhomologous sequences, such as those comprising the DNA or RNA or a vector into which the homologous sequence had been inserted for propagation. In either instance, the probe as presented as an analytical reagent will exhibit detectable hybridization at one or more points with sample nucleic acids of interest. Linear or circular single-stranded polynucleotides can be used as the probe element, with major or minor portions being duplexed with a complementary polynucleotide strand or strands, provided that the critical homologous segment or segments are in single-stranded form and available for hybridization with sample DNA or RNA. Useful probes include linear or circular probes wherein the homologous probe sequence is in essentially only single-stranded form (see particularly, Hu and Messing, Gene, 17:271 (1982)).

The nucleic acid probe of the present invention can be used in any conventional hybridization technique. As improvements are made and conceptually new formats are developed, such can be readily applied to the present probes. Conventional hybridization formats which are particularly useful include those wherein the sample nucleic acids or the polynucleotide probe is immobilized on a solid support (solid-phase hybridization) and those wherein the polynucleotide species are all in solution (solution hybridization).

In solid-phase hybridization formats, one of the polynucleotide species participating in hybridization is fixed in an appropriate manner in its single-stranded form to a solid support. Useful solid supports are well known in the art and include those which bind nucleic acids either covalently or noncovalently. Noncovalent supports which are generally understood to involve hydrophobic bonding include naturally occurring and synthetic polymeric materials, such as nitrocellulose, derivatized nylon and fluorinated polyhydrocarbons, in a variety of forms such as filters, beads or solid sheets. Covalent binding supports (in the form of filters, beads or solid sheets, just to mention a few) are also useful and comprise materials having chemically reactive groups or groups, such as dichlorotriazine, diazobenzyloxymethyl, and the like, which can be activated for binding to polynucleotides.

It is well known that noncovalent immobilization of an oligonucleotide is ineffective on a solid support, for example, on nitrocellulose paper. The present invention also describes novel methods of oligonucleotide immobilization. This is achieved by phosphorylation of an oligonucleotide by a polynucleotide kinase or by ligation of the 5'-phosphorylated oligonucleotide to produce multi-oligonucleotide molecules capable of immobilization. The conditions for kinase and ligation reaction have been described in standard text books, e.g., Molecular Cloning, T. Maniatis, E.F. Fritsch and J. Sambrook, Cold Spring Harbor Laboratory, (1982), pages 1-123.

A typical solid-phase hybridization technique begins with immobilization of sample nucleic acids onto the support in single-stranded form. This initial step essentially prevents reannealing of complementary strands from the sample and can be used as a means for concentrating sample material on the support for enhanced detectability. The polynucleotide probe is then contacted with the support and hybridization detected by measurement of the label as described herein. The solid support provides a convenient means for separating labelled probe which has hybridized to the sequence to be detected from that which has not hybridized.

Another method of interest is the sandwich hybridization technique wherein one of two mutually exclusive fragments of the homologous sequence of the probe is immobilized and the other is labelled. The presence of the polynucleotide sequence of interest results in dual hybridization to the immobilized and labelled probe segments. See Methods in Enzymology , 65:468 (1980) and Gene, 21:77-85 (1983) for further details.

For the present invention, the immobile phase of the hybridization system can be a series or matrix of spots of known kinds and/or dilutions of denatured DNA. This is most simply prepared by pipetting appropriate small volumes of native DNA onto a dry nitrocellulose or nylon sheet, floating the sheet on a sodium hydroxide solution to denature the DNA, rinsing the sheet in a neutralizing solution, then baking the sheet to fix the DNA. Before DNA:DNA hybridization, the sheet is usually treated with a solution that inhibits non-specific binding of added DNA during hybridization.

This invention involves the labeling of whole genomic DNA, whole nucleic acids present in cells, whole cell lysate, or unlysed whole cells. Once the labeled material is prepared, it can be used for the detection, i.e., the presence or absence of certain specific genomic sequences by specific nucleic acid hybridization assays.

One method according to the invention involves the separation of cells from a human sample or the human sample directly is treated by mixing with a photochemically reactive nucleic acid binding intercalating ligand. The mixture is incubated depending on the type of the sample. If the sample is lysed cells or nucleic acids, it is incubated for a period between a few seconds to five minutes and when whole cells or partially lysed cells are used, incubation between two minutes to two hours is employed. After the mixing and incubation, the whole sample mixture is irradiated at a particular wavelength for the covalent interaction between the photochemically reactive DNA binding ligand and the test sample. Then this labeled material is hybridized under specific hybridization conditions with a specific probe.

After the hybridization, the unreacted unhybridized labeled test sample is removed by washing. After the washing, the hybrid is detected through the label carried by the test sample, which is specifically hybridized with a specific probe.

The present invention is surprising since in a human genomic sample the amount of a single copy gene is very low, for example, if a restriction fragment of one thousand base pair is the region of hybridization, the probability of such sequence in the whole human genomic sample is one in a million. This conclusion has been derived by assuming from the literature that a human genomic sample has $3 \times 10^9$ base pairs and 1000 base pairs will be 1/3,000,000 of that number. Automatically, in a sample of human DNA containing approximately five to ten micrograms of nucleic acids, only 5 to 10 picogram of the corresponding sequences is available and labeling the vast majority of the non-specific DNA should produce more background than the true signal. But after the reaction, it is surprising to observe that the results are not only specific, but also of unexpected higher sensitivity.

Without wishing to be bound by any particular theory of operability, the reason for the unexpected sensitivity may be due to the formation of a network of non-specific nucleic acid hybrids bound to the specific hybrid, thus amplifying the amount of the signal. As has been shown in a typical example, a 19 nucleotide long specific sequence containing plasmid is immobilized and hybridized with 5 microgram equivalent of a test sample which is labeled photochemically and one detects very easily the signal resulted from such hybrid. This could not have been accomplished by any other technique because of the problems associated with the labeling method.

The present invention relates to a novel hybridization technique where probes are immobilized and an eukaryotic nucleic acid sample is labeled and hybridized with immobilized unlabeled probe. A surprising characteristic of the invention is the ability to detect simple or multiple copy gene defects by labeling the test sample. Since there is no requirement for an excess of labeled hybridizing sequence, the present method is more specific. In the present invention, simultaneous detection of different gene defects can be easily carried out by immobilizing specific probes.

For example, using the present invention, one can immobilize oligonucleotide probes specific for genetic defects related to sickle cell anemia and probes for alpha-thalassemias on a sheet of nitrocellulose paper, label the test sample and hybridize the labeled test sample with the immobilized probes. It is surprising that partially purified or unpurified nucleic acid samples (cell lysate or whole cell) can be photochemically labeled with sensitive molecules without affecting the specific hybridizability.

The present invention is also directed to detecting eukaryotes (protists) in samples from higher organisms, such as animals or humans.

Eukaryotes include algae, protozoa, fungi and slime molds.

The term "algae" refers in general to chlorophyll-containing protists, descriptions of which are found in G.M. Smith, Cryptogamic Botany, 2nd ed. Vol. 1, Algae and Fungi , McGraw-Hill, (1955).

Eukaryotic sequences according to the present invention includes all disease sequences except for bacteria and viruses. Accordingly, genetic diseases, for example, would also be embraced by the present invention. Non-limiting examples of such genetic diseases are as follows:

| Area Affected | Diseases |
|---|---|
| Metabolism | Acute intermittent porphyria |
| | Variegate porphyria |
| | $alpha_1$-antitrypsin deficiency |
| | Cystic fibrosis |
| | Phenylketonuria |
| | Tay-Sachs disease |
| | Mucopolysaccharidosis I |
| | Mucopolysaccharidosis II |
| | Galactosaemia |
| | Homocystinuria |
| | Cystinuria |
| | Metachromic leucodystrophy |
| Nervous System | Huntington's chorea |
| | Neurofibromatosis |
| | Myotonic dystrophy |
| | Tuberous sclerosis |
| | Neurogenic muscular atrophies |
| Blood | Sickle-cell anaemia |
| | Beta-thalassaemia |
| | Congenital spherocytosis |
| | Haemophilia A |
| Bowel | Polyposis coli |
| Kidney | Polycystic disease |

| | |
|---|---|
| Eyes | Dominant blindness |
| | Retinoblastoma |
| Ears | Dominant early childhood deafness |
| | Dominant otosclerosis |
| Circulation | Monogenic hypercholesterolaemia |
| Blood | Congenital spherocytosis |
| Teeth | Dentinogenisis imperfecta |
| | Amelogenisis imperfecta |
| Skeleton | Diaphysial aclasia |
| | Thanatophoric dwarfism |
| | Osteogenes imperfecta |
| | Marfan syndrome |
| | Achondroplasia |
| | Ehlers-Danlos syndrome |
| | Osteopetrosis tarda |
| | Cleft lip/palate |
| Skin | Ichthyosis |
| Locomotor | Muscular dystrophy |

A nucleic acid probe in accordance with the present invention is a sequence which can determine the sequence of a test sample. The probes are usually DNA, RNA, mixed copolymers of ribo-and deoxyribonucleic acids, oligonucleotides containing ribonucleotides or deoxyribonucleotide residues or their modified forms. The sequence of such a probe should be complementary to the test sequence. The extent of complementary properties will determine the stability of the double helix formed after hybridization. The probe can also have covalently linked non-complementary nucleic acids. They can serve as the sites of the labeling reaction.

The nucleic acid is preferably labeled by means of photochemistry, employing a photoreactive DNA-binding furocoumarin or a phenanthridine compound to link the nucleic acid to a label which can be "read" or assayed in conventional manner, including fluorescence detection.

One use of the present invention is the identification of bacterial species in biological fluids. In one application, samples of urine from subjects having or suspected of having urinary tract infections can provide material for the preparation of labelled DNA(s) or RNAs, while a solid support strip, e.g., made of nitrocellulose or nylon, can contain individual dots or spots of known amounts of denatured purified DNA from each of the several bacteria likely to be responsible for infection.

The format of labeled unknown and unlabeled probes, which is the converse of standard schemes, allows one to identify among a number of possibilities the species of organism in a sample with only a single labeling. It also allows simultaneous determination of the presence of more than one distinguishable bacterial species in a sample (assuming no DNA in a mixture is discriminated against in the labeling procedure). However, it does not allow in a simple way, better than an estimate of the amount of DNA (and, therefore, the concentration of bacteria) in a mixed sample. For such quantitation, sample DNA is immobilized in a series of dilution spots along with spots of standard DNA, and probe DNAs are labeled.

A urinary tract infection is almost always due to monoclonal growth of one of the following half dozen kinds of microorganism: Escherichia coli (60-90% of UTI), Proteus spp. (5-20% of UTI), Klebsiella spp (3-10% of UTI), Staphylococcus spp. (4-20% of UTI), Streptococcus spp. (2-5% of UTI). Pseudomonas and some other gram negative rods together account for a low percentage of UTI. A common contaminant of urine samples that is a marker of improper sample collection is Lactobacillus.

The concentration of bacteria in a urine sample that defines an infection is about $10^5$ per milliliter.

The format for an unlabeled probe hybridization system applicable to urinary tract infections is to have a matrix of DNAs from the above list of species, denatured and immobilized on a support such as nitrocellulose, and in a range of amounts appropriate for concentrations of bacterial DNAs that can be expected in samples of labelled unknown.

Standard hybridization with biotinylated whole genome DNA probes takes place in 5-10 ml, at a probe concentration of about 0.1 µg/ml; hybridization of probe to a spot containing about 10 ng denatured DNA is readily detectable. There is about 5 fg of DNA per bacterial cell, so that for a sample to contain 1 µg of labelled DNA, it is necessary to collect $2 \times 10^8$ bacteria. If an infection produces urine having approximately $10^5$ bacteria/ml, then bacterial DNA to be labeled from a sample is concentrated from 2000 ml. If more than 10 ng unlabeled probe DNA is immobilized in a dot, for example, 100 ng or 1 µg, or if the hybridization volume is reduced, then the volume of urine required for the preparation of labeled unknown is approximately a few tenths of a ml.

A strip of dots containing immobilized, denatured, unlabelled probe DNAs could have the following configuration:

| | 1 µg | 10 ng | 100 pg |
|---|---|---|---|
| Escherichia | o | o | o |
| Proteus | o | o | o |
| Klebsiella | o | o | o |
| Staphylococcus | o | o | o |
| Streptococcus | o | o | o |
| Pseudomonas | o | o | o |
| Lactobacillus | o | o | o |

This procedure involves the labeling of DNA or RNA in a crude cell lysate. Ideally, preparation of labeled sample DNA or RNA will accommodate the following points:

(1) bacteria will be concentrated from a fluid sample by centrifugation or filtration;

(2) bacteria will be lysed under conditions sufficient to release nucleic acids from the most refractory of the organisms of interest;

(3) the labeling protocol will not require purification of labeled nucleic acids from unincorporated precursors, nor the purification of nucleic acids prior to labeling;

(4) the labeling protocol will be sufficiently specific for DNA and/or RNA that proteins, lipids and polysaccharides in the preparation will not interfere with hybridization nor read-out.

In the present invention, there is provided a method for efficiently and rapidly lysing whole cells, including Gram positive bacteria. The method involves contacting cells, e.g., whole cells, with an alkali, e.g., sodium or potassium hydroxide solution in a concentration of 0.1 to 1.6 Normal.

The important features of the present lysis protocol are its relative simplicity and speed. It employs a common chemical that requires no special storage conditions and it lyses even Gram positive organisms with high efficiency, while preserving the properties of the DNA that are important for subsequent steps in the photochemical labeling process.

For the present invention, the immobile phase of the hybridization system can be a series or matrix of spots of known kinds and/or dilutions of denatured DNA. This is most simply prepared by pipetting appropriate small volumes of native DNA or oligonucleotides onto a dry nitrocellulose or nylon sheet, floating the sheet on a sodium hydroxide solution to denature the DNA, rinsing the sheet in a neutralizing solution, then baking the sheet to fix the DNA. Before DNA:DNA hybridization, the sheet is usually treated with a solution that inhibits non specific binding of added DNA during hybridization.

The invention will be further described in the following non-limiting examples wherein parts are by weight unless otherwise expressed.

## Examples

### Example 1: Preparation of Labelling Compound

The preparation of the labeling compound required 1-amino-17-N-(Biotinylamido)-3,6,9,12,15 pentaoxaheptadecane. This compound was prepared in the following four steps:

(a) 3,6,9,12,15 pentaoxapheptadecane 1,17-diol ditosylate was synthesized.
(b) 1,17-dipthalimido derivative of 3,6,9,12,15 pentaoxaheptadecane was prepared.
(c) 1,17-diamino derivative of 3,6,9,12,15 pentaoxaheptadecane was prepared.
(d) 1-amino, 17-biotinylamido derivative of 3,6,9,12,15 pentaoxaheptadecane was prepared.

### Example 1(a): Preparation of 3,6,9,12,15-Pentaoxaheptadecane-1,17-diol Ditosylate

To a stirred solution containing 50 g of hexaethylene glycol (0.177 mol) and 64 ml of triethylamine - (39.36 g, 0.389 mol) in 400 ml of $CH_2Cl_2$ at 0°C was added dropwise a solution containing 73.91 g of p-toluenesulfonyl chloride (0.389 mol) in 400 ml of $CH_2Cl_2$ over a 2.5 hour period. The reaction mixture was then stirred for one hour at 0°C and then heated to ambient temperature for 44 hours. The mixture was then filtered and the filtrate was concentrated in vacuo. The resulting heterogeneous residue was suspended in 500 ml of ethyl acetate and filtered. The filtrate was then concentrated in vacuo to a yellow oil which was triturated eight times with 250 ml portions of warm hexane to remove unreacted p-toluenesulfonyl chloride. The resulting oil was then concentrated under high vacuum to yield 108.12 g of a yellow oil (quantitative yield).

Analysis: Calculated for $C_{28}H_{42}O_{11}S_2$
Calc.: C, 52.87; H, 6.48.
found: C, 52.56; H, 6.39.
PMR: (60 MHz, $CDCl_3$) $\delta$ : 2.45 (s, 6H); 3.4-3.8 (m, 20H); 4.2 (m, 4H); 7.8 (AB quartet, J=8Hz, 8H).
IR: (neat) $cm^{-1}$: 2870, 1610, 1360, 1185, 1105, 1020, 930, 830, 785, 670.

### Example 1(b): Preparation of 1,17-Diphthalimido-3,6,9,12,15-pentaoxaheptadecane

A stirred suspension containing 108 g of 3,6,9,12,15-pentaoxaheptadecane-1,17-diol ditosylate (0.183 mol), 74-57 g of potassium phthalimide (0.403 mol), and 700 ml of dimethylacetamide was heated at 160-170°C for 2 hours and was then cooled to room temperature. The precipitate was filtered and washed with water and acetone to yield 53.05 g of product as a white powder which was dried at 55°C (0.1 mm). mp 124-126°C.

A second crop of product was obtained from the dimethylacetamide filtrate by evaporation in vacuo and the resulting precipitate with was successively washed ethyl acetate, water, and acetone. The resulting white powder was dried at 55°C (0.1 mm) to yield an additional 9.7 g of product. mg 124.5-126.5°C. The combined yield of product was 62.82 g (68% yield).

Analysis: (For first crop)
Calculated for $C_{28}H_{52}N_2O_{9}.1/2H_2O$
Calc.: C, 61.19; H, 6.05; N, 5.09.
found: C, 61.08; H. 6.15; N, 5.05.

(For second crop)
Calculated for $C_{28}H_{52}N_2O_9$
Calc.: C, 62.21; H, 5.97; N, 5.18.
found: C, 61.78; H, 6.15; N, 5.13.
PMR: (60 MHz, dmso-$d_6$) $\delta$ : 3.5 (s, 8H); 3.6 (s, 8H); 3.8 (bt, J = 3Hz, 8H): 8.1 (s, 8H).
IR: (KBr) $cm^{-1}$: 2890, 1785, 1730, 1400, 1100, 735.

Example 1(c): Preparation of 1,17-Diamino-3,6,9,12,15-Pentaoxaheptadecane

A solution containing 60 g of 1,17-diphthalimido-3,6,9,12,15-pentaoxaheptadecane (0.118 mol), 14.8 g of hydrazine hydrate (0.296 mol), and 500 ml of ethanol were heated with mechanical stirring in a 100°C oil bath for three hours. The mixture was then cooled and filtered. The resultant filter cake was washed four times with 300 ml portions of ethanol. The combined filtrates were concentrated to yield 32.35 g of a yellow apaque glassy oil. The evaporative distillation at 150-200°C (0.01 mm) gave 22.82 g of a light yellow oil - (69% yield). lit. b.p. 175-177°C (0.07 mm).
PMR: (60 MHz, $CDCl_3$) $\delta$ : 1.77 (s, 4H, $NH_2$); 2.85 (t, J = 5Hz, 4H); 3.53 (t, J = 5Hz, 4H); 3.67 (m, 16H).
IR: ($CHCl_3$) $cm^{-1}$: 3640, 3360, 2860, 1640, 1585, 1460, 1350, 1250, 1100, 945, 920, 870.
Mass Spectrum: (EI) m/e = 281.2 (0.1%, M + 1).
(FAB) m/e = 281.2 (100%, M + 1).
Analysis: For $C_{12}H_{28}N_2O_5.1/2\ H_2O$
Calc.: C, 49.80, H, 10.10; N, 9.68.
found: C, 50.36, H, 9.58; N, 9.38.
Literature Reference: W. Kern, S. Iwabachi, H. Sato and V. Bohmer, Makrol. Chem., 180, 2539 (1979).

Example 1(d): Preparation of 1-Amino-17-N-(Biotinylamido)-3,6,9,12,15-pentaoxaheptadecane

A solution containing 7.2 g of 1,17-diamino-3,6,9,12,15-pentaoxaheptadecane (25 mmol) in 75 ml of DMF under an argon atmosphere was treated with 3.41 g of N-succinimidyl biotin (10 mmol) added in portions over 1.0 hour. The resulting solution was stirred for four hours at ambient temperature. TLC ($SiO_2$, 70:10.1 $CHCL_3$-$CH_3OH$-conc. $NH_4$ OH) visualized by dimethylaminocinnamaldehyde spray reagent showed excellent conversion to a new product (Rf = 0.18). The reaction mixture was divided in half and each half was absorbed onto $SiO_2$ and flash-chromatographed on 500 g of $SiO_2$-60 (230-400 mesh) using a 70:10.1 $CHCl_3$-$CH_3OH$-conc. $NH_4OH$ solvent mixture. Fractions containing the product were polled and concentrated to a yield 2.42 g of a gelatinous, waxy solid. The product was precipitated as a solid from isopropanol-ether, washed with hexane, and dried at 55°C (0.1 mm) to give 1.761 g of a white powder (35% yield).
Analysis: Calculated for $C_{22}H_{42}N_4O_7S.3/2\ H_2O$:
C, 49.51; H, 8.50; N. 10.49.
found: C, 49.59; H, 8.13; N, 10.39.
PMR: (90 MHz, dmso-$d_6$) $\delta$ : 1.1-1.7 (m, 6H); 2.05 (t, J = 7Hz, 2H);
2.62 (t, J = 4Hz, 1H); 2.74 (t, J = 4Hz, 1H); 3.0-3.4 (m, 14H).
3.50 (s, 14H); 4.14 (m, 1H); 4.30 (m, 1H); 6.35 (d, J = 4Hz, 1H); 7.80 (m, 1H).
CMR: (22.5 MHz, dmso-$d_6$) $\delta$ : 25.2, 28.0, 28.2, 35.1, 40.6, 55.3, 59.2, 61.1, 69.6, 69.8, 71,2, 162.7, 172.1.
IR: (KBr) $cm^{-1}$: 2900, 2850, 1690, 1640, 1580, 1540, 1450, 1100.
Mass Spectrum (FAB) m/e: 507.3 (M + 1, 56%)

Example 2: Preparation of 4'-Biotinyl-PEG-4,5'-dimethylangelicin

A solution of 203 mg of 1-amino-17-N-(biotinylamido)-3,6,9.12,15-pentaoxaheptadecane (0.4 mmol) in 1 ml of DMF under an argon atmosphere was treated with 78 mg of N,N-carbonyldimidazole (0.48 mmol). The resulting mixture was stirred for four hours and was then treated with 55 mg of 4'-aminomethyl-4,5'dimethylingelicin hydrochloride (0.2 mmol), 140 µl of diisopropylethylamine, and 100 µl of DMF. The resulting

mixture was stirred overnight at 50°C. The mixture was then evaporated onto $SiO_2$ in vacuo and the resultant impregnated solid flash was chromatographed on 60 g of $SiO_2$ (230-400 mesh) eluted with 1.5 liters of 7% $CH_3$-$CHCl_3$ followed by 1 liter of 10% $CH_3OH$-$CHCl_3$. Fractions containing the product were pooled and concentrated to yield 72 mg of a glassy solid (47% yield).

PMR: (90 MHz, dmso-$d_6$): $\delta$ 1.1-1.8 (m, 6H); 2.04 (bt, J=7Hz, 2H); 2.5 (s, 6H); 2.56 (m, 1H); 2.74 (bd, J=4Hz, 1H); 2.8-3.4 (m, 14H); 3.40 (m, 14H); 4.14 (m, 1H); 4.25 (m, 1H); 4.40 (bd, J=6Hz, 2H); 6.5 (m, 1H); 6.35 (s, 1H); 7.02 (s, 1H); 7.45 (d, J=8Hz, 1H); 7.62 (d, J=8Hz, 1H); 7.80 (m, 1H).

CMR: (22.5 MHz, dmso-$d_6$) $\delta$ : 11.9, 18.9, 25.3, 28.2 28.3, 33.4, 35.2, 55.4, 59.2, 61.0, 69.2, 69.6, 69.8, 70.0, 89.0, 107.8, 112.0, 113.1, 114.3, 120.6, 121.6, 153.6, 154.4, 155.6, 157.9, 159.5, 162.7, 172.1.

Literature Reference: F. Dall'Acqua, D. Vedaldi, S. Caffieri, A. Guiotto, P. Rodighiero, F. Baccichetti, F. Carlassare and F. Bordin, J. Med. Chem., 24, 178 (1981).

## Example 3: Colorimetric or Chemiluminescent Detection of the Nucleic Acid Hybrids

### Example 3(a): Colorimetric Detection

Colorimetric detection of the biotinylated hybrids is carried out following the procedure and kit developed by Bethesda Research Laboratories (BRL), Gaithersburg, Maryland 20877, U.S.A. The procedure is described in detail in a manual supplied with a kit by BRL, entitled "Products for Nucleic Acid Detection", "DNA Detection System Instruction Manual", Catalogue No. 8239SA.

### Example 3(b): Chemiluminescent Detection

Chemiluminescent detection of the biotinylated hybrids is identical to the above method: the filters with the hybrids are saturated with BSA (bovine serum albumin) by immersing the paper in 3% BSA at 42°C for 20 minutes. Excess BSA is removed by taking the paper out of the container, and blotting it between two pieces of filter paper. The paper is then incubated in a solution containing Streptavidin (0.25 mg/ml, 3.0 ml total volume), for 20 minutes at room temperature. It is then washed three times with a buffer containing 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 2mM $MgCl_2$, 0.05% "TRITON X-100". Next the filter is incubated with biotinylated horseradish peroxidase (0.10 mg/ml) for 15 minutes at room temperature. This is followed by three washings with 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$ and 0.05% Triton X-100, and one washing with 10 mM Tris (pH 8.0) buffer. Chemiluminescent activation is conducted in two ways. (1) Spots are punched out and the discs containing the DNA are placed in a microtiter plate with wells that are painted black on the sides. After the punched paper circles are placed in the microtiter place wells, 0.8 ml buffer containing 40 mM Tris and 40 mM ammonium acetate (pH 8.1) is added to each well. Then 10 µl of 1:1 mixture of 39 mM Luminol (in DMF) and 30 mM $H_2O_2$ (in water) is added. Light emission is recorded on a "POLAROID" instant film by exposing it directly in the film holder. Alternatively (2), the paper is soaked in a solution containing 1:1 mixture of 0.5 mM Luminol and $H_2O_2$ and wrapped with a transparent "SARAN WRAP". The light emission is recorded on a " POLAROID" film as above.

## Example 4: General Method of Labeling the Test Sample Nucleic Acids

High molecular weight DNA from a patient's sample is isolated by a method described in U.S.P. 4,395,486 (Wilson et al), the entire contents of which are incorporated by reference herein. The nucleic acid is dissolved in 10 mM borate buffer (pH 8.0) to a final concentration of approximately 20 µg/ml. To the nucleic acid solution "angelicin-peg-biotin" in aqueous solution is added to a final concentration of 10 µg/ml. The mixture is then irradiated at long wavelength irradiation for about 60 minutes using a black ray UVL 56 lamp. The product is ready for hybridization without purification. However, the product can be purified by dialysis or alcohol precipitation (U.S.P. 4,395,486) as is usually followed for nucleic acids.

Instead of nucleic acids, whole cell lysate can also be labeled following an identical procedure. The lysis is conducted by boiling the cells with 0.1 N sodium hydroxide, followed by neutralization with hydrochloric acid.

When whole cells are used, the mixture of "PEG-ang-bio" and cells are incubated for at least 60 minutes prior to irradiation for efficient transport of the ligands. Many different variations of the above described methods can be adopted for labeling.

Example 5:

Alpha-thalassemia is associated with gene deletion. The detection of gene deletion by hybridization in a dot/slot blot format requires that the total amount of sample and its hybridizability are accurately known. Since the beta-globin gene is a single copy gene, simultaneous hybridization of a sample with beta-globin and alpha-globin and their relative amounts will indicate the amount of alpha-globin with the sample.

The format and hybridization conditions are the same as Rubin and Kan, supra, except probes, not test DNA, is immobilized. Hybridization conditions are also similar. The detection is done by using the BRL kit described supra following BRL's specifications.

The hybridization detection process are conducted in three steps as follows:

Step 1: Immobilization of the Probes

As described in Rubin and Kan, supra, 1.5 kb PstI fragment containing alpha$_2$ globin gene is used as a probe for alpha-thalassemia and for the beta-globin gene a 737 base pair probe produced by the digestion of pBR beta Pst (4.4 kb) is used. The beta-globin gene probe has been described in U.S.P. 4,395,486 (column 4). For the detection of gene deletion related to alpha-thalassemia, the amount of starting nucleic acid, hybridization efficiency and control samples are needed. The present invention avoids these problems by simultaneous hybridization with a single copy essential gene (e.g., beta-globin gene) when similar amounts of probes are immobilized side by side, labeled sample is hybridized, relative strength of signal intensity is a measure of relative amount of gene dosage present in the sample.

The probes (0.5, 1, 3 and 5 µg per 100 µl) are suspended in 10 mM tris HCl (pH 7) buffer, denatured with 20 µl 3 M sodium hydroxide, at 100°C, for 5 minutes, an equivalent volume of 2 M ammonium acetate, pH 5.0 is added to neutralize the solution, immediately after neutralization the probes for beta-and alpha-globin genes are applied in parallel rows to nitrocellulose filter paper under vacuum in a slot blot manifold, purchased from Scleicher and Schuell, (Keeni, New Hampshire, U.S.A.). The filter is then dried in vacuum at 80°C for 60 minutes. It is then prehybridized for 4 hours in a mixture containing 50 mM sodium phosphate (pH 7) 45 mM sodium citrate, 450 mM sodium chloride, 50% (v/v) formamide, 0.2% each (w/v) of polyvinyl pyrrolidine, "FICOLL 400" and bovine serum albumin and 0.2 mg/ml alkali boiled salmon sperm DNA and 0.15 mg/ml yeast RNA.

Step 2: Labeling of the Test Sample

This was described above.

Step 3: Hybridization

The nitrocellulose strip containing the immobilized probes are hybridized with the labeled test sample in plastic bags (e.g., "SEAL-A-MEAL", "SEAL and SAVE", etc.). Hybridization solution is the same as prehybridization solution plus 10% dextran sulphate. Hybridization is done at 42°C for 16 hours. After hybridization detection of biotin is conducted with a kit and procedure supplied by Bethesda Research Laboratory, Maryland, U.S.A., (catalogue No. 8239SA). Results of relative intensity of alpha-and beta-regions are used to estimate the extent of deletion of alpha-globin genes:

No signal on the alpha-globin side: all 4 alpha-globin genes missing.

Signal on the alpha-globin side is half as strong as on the corresponding beta-side: 3 alpha-globin genes missing.

Signals on alpha and beta side equivalent: 2 alpha-globin genes missing.

Signals on alpha side is stronger than the corresponding beta side (2 alpha = 3 beta): 1 alpha-globin gene missing.

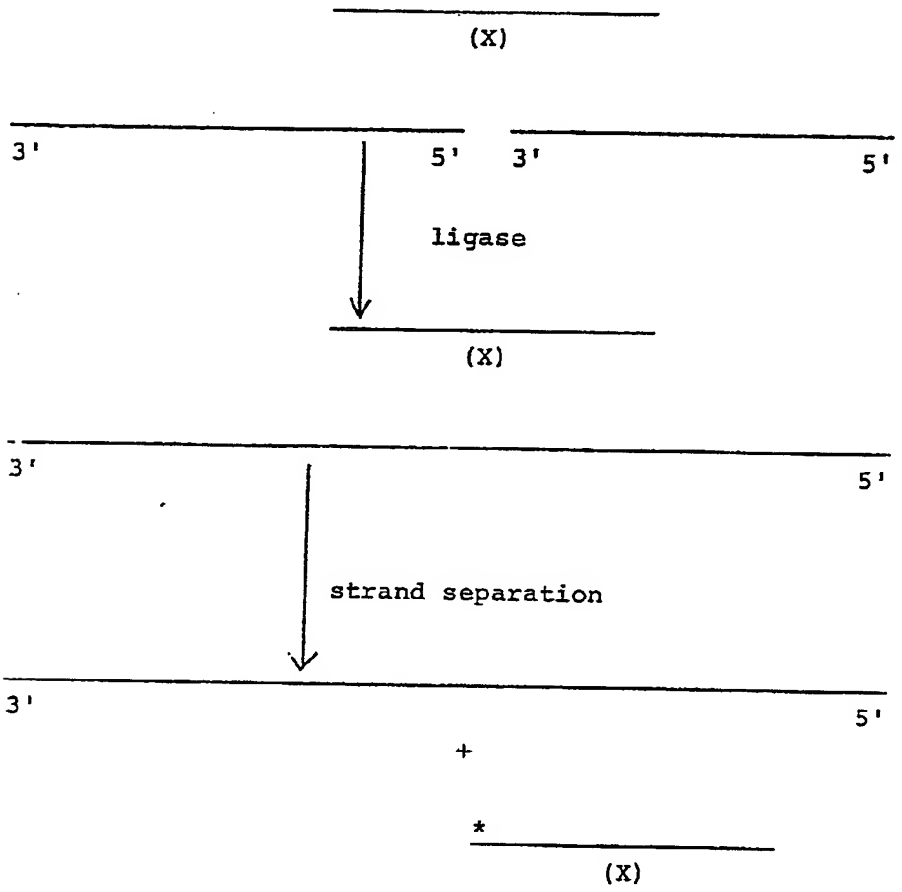
Example 6: Immobilization of an Oligonucleotide Sequence Specific for Hemoglobin Mutation

It is known that an oligonucleotide cannot be easily immobilized onto nitrocellulose paper by a simple adsorption process. The present invention encompasses three different methods to incorporate an oligonucleotide sequence into a larger molecule capable of adsorption.

Method 1: Two oligonucleotides, one a 43mer and the other a 16-mer, have been chemically synthesized in an automated synthesizer (Applied Biosystem 380B) by the phosphoramidite-method and phosphorylated at the 5' end by a T4-polynucleotide kinase mediated process according to Maniatis et al, Molecular Cloning, page 122. These oligonucleotides contain a segment of a 19 nucleotide long sequence specific for the detection of the mutation associated with sickle cell anemia.

43mer A & S (A = normal globin gene; S = sickle globin gene) were kinased according to Maniatis et al, Molecular Cloning, page 122, in two separate reactions, namely, one with $^{32}$P-ATP and one with no radioactive label. 0.4 µg $^{32}$P-43mer and 0.6 mg cold 43mer were mixed and purified on a spun column (G-25med in TE (Tris EDTA buffer)) to a final volume of 40 µl. Two dilutions were spotted on S & S (Schleicher & Schuell) nitrocellulose and nytran (nylon) membranes at 50 and 0.5 ng.

Method 2: The phosphorylated oligonucleotide products of method 1 were further elongated by making multimers of sequences by a ligase mediated process. The principle is described as follows:



The product being of a higher molecular weight than an oligonucleotide it should be immobilizable by adsorption on to a nitrocellulose paper.

Aqueous solutions containing 4µg of $^{32}$P43mer and 3.7 µg 16mer linker (X) were mixed and dried under vacuum. 6 mg of cold kinased 43mer was added and the sample was heated to 55°C and cooled slowly to 0°C to anneal. Ligation was carried out in 20 µl total reaction volume with 800 units of ligase (Pharmacia) at 15°C for 4 hours. 1 mg (2 µl) was purified on a spun column (G-25med in TE) to a final volume of 40 µl. Two dilutions were spotted on nitrocellulose nylon membranes at 50 and 0.5 ng.

Method 3: The same as method 2, but ligation was not conducted. Instead of ligation, cross linking was conducted with an intercalator to keep the double stranded regions intact. Hence, the cross linked molecule will have several oligonucleotide sequences covalently linked to each other.

2 µg of $^{32}$P43mer (for sequence P-50) was added to 2.9 mg of a 16mer (for sequence P-50) linker and purified on a spun column.(G25med in TE) to a final volume of 40 µl. 6 mg of kinased 43mer was added and the samples were heated to 55°C and cooled slowly to 0°C to anneal. 25 µl of intercalation compound aminomethyltrioxsalen was added and the sample was irradiated for 30 minutes on ice in 500 µl total 10 mM borate buffer pH 8.2 with a long wave UV lamp model (UVL-21, λ = 366 nM).

The probes modified by all three methods were then immobilized on to nitrocellulose and nylon paper and hybridized with labelled oligonucleotides. The results indicate that the sequence are immobilizable and hybridization fidelity remains intact.

Two dilutions of the products of methods 1 to 3 were spotted on nitrocellulose and nylon membranes at 50 and 0.5 ngs.

Whole filters were baked for 30 minutes in 80°C vacuum oven and prehybridized in blotto (5% nonfat dry milk, 6XSSC, 20 mM Na-pyrophosphate) for 30 minutes in 50°C oven.

Hybridization was carried out with primer extended 19'A & 19S' probes at 50°C for one hour (3 strips/probe).

Filters were stringently washed for 15 minutes at room temperature in 6XSSC with slight agitation and 2 x 10 minutes at 57°C.

Air-dried filters were place on Whatman paper and autoradiographed at -70°C overnight.

The results presented in Fig. 1 surprisingly indicate specific hybridization are obtained by immobilizing oligonucleotide probes.

Example 7: Hybridization with labeled genomic DNA for Non Radioactive Detection

Human normal genomic (XX) DNA was photolabeled with "biotin-PEG-angelicin" (BPA) in 10 mM borate buffer pH 8.2 at a weight ratio of 0.3 to 1 (BPA:DNA) for 15 minutes on ice with a long wave UV lamp model UVL-21, λ = 366 nm. No purification is necessary.

Target DNA oligonucleotides were directly immobilized on S & S nitrocellulose in 1 µl aliquots at the following concentration, and then baked in an 80°C vacuum oven for 30 minutes. The amounts of the different immobilized probes are as follows:

| | | | | |
|---|---|---|---|---|
| 43-mer | (A) | - | Kinased (method 1) | 200 ng |
| 43-mer | (A) | | | 200 ng |
| 43-mer | (S) | - | Kinased (method 1) | 200 ng |
| 43-mer | (S) | | | 200 ng |
| M1319Ass | | | | 50 ng |
| M1319Sss | | | | 50 ng |
| M13737Ass | | | | 50 ng |
| BRL Commercially biotinylated DNA | | | | 200 pg |
| pUC19 | | | | 50 ng |

43-merA:
5' CTGCT,AATCTTAAGGAT,AAT,CTCCTGAGGAGAAGTCT GCT,AATCTTAA5,GGAT,AAT,CT 3'

• = T for 43-mer S
16-mer (Common to both A and S)
3' -TTAGAATTCCTAAATT-5'

Filters were prehybridized in blotto (5% nonfat dry milk, 6XSSC, 20 mM Na-pyrophosphate) for 30 minutes in a 45°C $H_2O$ bath.

All 4 strips were hybridized in 2 mls solution containing 2 µg labeled XX DNA containing normal beta-globin gene (hybridization solution was blotto with 10% PEG) for 2 hours in 45°C in a $H_2O$ bath.

A stringency wash was carried out as follows:

1 x 20' at room temperature in 6XSSC

2 x 20' at temperatures indicated in Fig. 2 with very little agitation.

50 ml centrifuge tubes were used for elevated temperature washes. Results are shown in Fig. 2.

Detection of biotin in the hybrid was carried out according to the Bethesda Research Laboratory, Bethesda, Maryland, U.S.A., manual using their kit for biotin detection. The results indicated specific hybridization.

Example 8: Immobilization of Whole Genomic DNA As Probes

Tens of milligram to gram amounts of DNA were prepared in the following manner from bacterial cells harvested from fermentor cultures. Bacteria were collected by centrifugation from 10 liter nutrient broth cultures grown in a New Brunswick Scientific Microferm Fermentor. Generally, cells in concentrated suspension were lysed by exposure to an ionic detergent such as SDS (Na dodecyl sulfate), then nucleic acids were purified from proteins and lipids by extraction with phenol and/or chloroform (J. Marmur, J. Mol. Biol., 3 , 208-218, 1961). RNA was removed from the nucleic acids preparation by treatment of the DNA solution with 0.2 mg/ml ribonuclease at 37°C, then DNA was precipitated from solution by the addition of two volumes of ethanol. Bacterial DNA redissolved from the precipitate in a low salt buffer such as TE (10 mM Tris-HCl, pH 7.5, 1mM $Na_2$ EDTA) was characterized with respect to purity concentration and molecular size, then approximately 1 microgram aliquots were denatured and immobilized as spots on nitrocellulose or nylon membranes for hybridization (Kafatos et al., Nucleic Acids, Res. 7 , 1541-1552, (1979)). Denaturation was accomplished by exposure of the DNA with approximately 0.1 N NaOH. After denaturation the solution was neutralized, then the membrane was rinsed in NaCl/Tris-HCl, pH 7.5, and dried.

Example 9: Processing of a Test Sample for Cellular DNA Labeling

Samples of urine, for example (although the following can equally apply to suspensions of material form gonorrhea-suspect swabs, from meningitis-suspect cerebrospinal fluid, from contamination-suspect water samples, etc.), are centrifuged or filtered to wash and concentrate any bacteria in the sample. The bacteria are then lysed by exposure to either (i) 2 mg/ml lysozyme or lysostaphin then exposure to approximately 90°C heat, (ii) 0.1 to 1.6 N NaOH, or (iii) 1% Na dodecyl sulfate. After (ii) NaOH, the cell lysate solution is neutralized before labelling; after (iii) detergent lysis, DNA labelling is preceded by removal of the SDS with 0.5 M K acetate on ice. Angelicin should be able to permeate intact cells so that DNA labeling can be accomplished before cell lysis. This in situ labeling simplifies the extraction procedure, as alkaline or detergent lysates can be incorporated directly into a hybridization solution.

Prior to hybridization, the labeled sample is denatured, and it should also preferably be reduced to short single stranded lengths to facilitate specific annealing with the appropriate unlabeled probe DNA. Methods of denaturation are known in the art. These methods include treatment with sodium hydroxide, organic solvent, heating, acid treatment and combinations thereof. Fragmentation can be accomplished in a control way may be heating the DNA to approximately 80°C in NaOH for a determined length of time, and this, of course, also denatures the DNA.

Example 10: Labeling of the Products of Example 9

(i) A test sample of about 10ml urine will contain $10^4$ or more infectious agents. After separation by centrifugation and washing, the pretreated cell lysate (step 2) was resuspended in 0.2 ml 10 mM sodium borate buffer (pH approximately 8). To this suspension, 10 µg of photolabelling reagent dissolved in ethanol

(10 mg/ml), was added and mixed by shaking on a vortex mixer. The mixture was then irradiated at 365 nm for 30 minutes with a UVGL 25 device at its long wavelength setting. The UVGL device is sold by UVP Inc., 5100 Walnut Grove Avenue, P.O. Box 1501, San Gabriel, CA 91778, U.S.A.

(ii) The sample was also labeled with N-(4-azido-2-nitrophenyl)-N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine (commercially available from BRESA, G.P.O. Box 498, Adelaide, South Australia 5001, Australia), following the procedure described by Forster et al (1985), supra for DNA.

(iii) When unlysed cells were used, the cell suspension in 0.2 ml 10 mM borate was incubated with the photoreagent for 1 hour prior to irradiation.

Example 11: Hybridization of the Products of Examples 8 and 10

Prior to hybridization, the membrane with spots of denatured unlabeled probe DNA was treated for up to 2 hours with a "prehybridization" solution to block sites in the membrane itself that could bind the hybridization probe. This and the hybridization solution, which also contained denatured labeled sample DNA, was comprised of approximately 0.9 M $Na^+$, 0.1% SDS, 0.1-5% bovine serum albumin or nonfat dry milk, and optionally formamide. With 50% formamide, the prehybridization and hybridization steps were done at approximately 42°C; without, the temperature was approximately 68°C. Prehybridized membranes can be stored for some time. DNA hybridization was allowed to occur for about 10 minutes or more, then unbound labeled DNA was washed from the membrane under conditions such as 0.018 M $Na^+$ (0.1 × SSC), 0.1% SDS, 68°C, that dissociate poorly base paired hybrids. After posthybridization washes, the membrane was rinsed in a low salt solution without detergent in anticipation of hybridization detection procedures.

Example 12: Detection of a Nucleic Acid Hybrid with Immunogold

Affinity isolated goat antibiotin antibody (purchased from Zymed Laboratories, San Francisco, California, U.S.A.) was adsorbed onto colloidal gold (20 nm) following the method described by its supplier (Janssen instruction booklet, Janssen Life Sciences Products, Piscataway, New Jersey, U.S.A.) and reacted with hybridized biotinylated DNA after blocking as in a colorimetric method. The signals were silver enhanced using a Janssen (B2340 BEERSE, Belgium) silver enhancement kit and protocol.

Example 13: Detection of Urinary Tract Infection in a Urine Sample

Urine samples were collected from a hospital where they were analyzed by microbiological methods and the results were kept secret until the hybridization diagnosis was conducted. Then they were compared ascertain the validity of the hybridization results.

1 ml of clinical sample (urine) suspected of UTI infection was centrifuged in a Brinkman micro centrifuge for 5 minutes. Then 0.1 ml of 1.2 N sodium hydroxide was added and the suspension was heated to 100°C to lyse the cells. The suspension was then diluted to 1 ml with 10 mM sodium borate buffer pH 8 and was neutralized with hydrochlorine acid to a pH of 7. To the solution, 50 µg "biotin-PEG-angelicin" (see Example 2) is added and the mixture was irradiated with a UVL 56 long wavelength UV lamp for 15 minutes. The irradiated sample (0.1 ml) was added to 3 ml 3XSSC of 5% nonfat dry milk 10% PEG with 0.2 M sodium pyrophosphate and hybridization was conducted with probes (whole genomic DNA) immobilized onto nitrocellulose paper at 68°C for 5 minutes to overnight. After hybridization detection was conducted according to Examples 3 or 12, the spots or the photographs were visually interpreted for the presence of specific bacteria in the test sample. A spot of human DNA was also present in the nitrocellulose paper for the detection of leucocytes. The presence of leucocytes was further verified with a common method using "LEUKOSTIX" (Miles Laboratories, Elkhart, Indiana, U.S.A.).

Typical results (Tables 1 and 2) indicate that the hybridization diagnosis produces similar results in a shorter time then the corresponding microbiological assays. The present invention not only provides information related to species identification, but also the leucocyte content in a clinical sample.

TABLE 1

DIAGNOSIS OF CLINICAL URINE SAMPLES

| * HOSPITAL DIAGNOSIS | APPLICANTS' HYBRIDIZATION RESULTS | DETECTION SYSTEM |
|---|---|---|
| NEG | NEG | GOLD |
| NEG | NEG | GOLD |
| NEG | NEG | GOLD |
| NEG | E.c.-M | CHEMI |
| NEG | E.c.-VW | GOLD |
| NEG | E.c.-VW | GOLD |
| S+, C- | NEG | GOLD |
| S+, C- | E.c.-S | CHEMI |
| S+, C- | E.c.-S, Kl.-M | CHEMI |
| S+, C- | NEG | GOLD |
| S+, C- | NEG | GOLD |
| S+, C- | NEG | GOLD |
| S+, C- | E.c.-VW | GOLD |
| S+, C- | NEG | GOLD |
| S+, C- | E.c.-VW | GOLD |
| S+, C- | NEG | GOLD |
| S+, C- | NEG | GOLD |
| 100,000/mL E.c. | E.c.-S | GOLD |
| 100,000/mL E.c. | E.c.-S | CHEMI |
| 100,000/mL E.c. | E.c.-W | GOLD |
| 50,000/mL E.c. | E.c.-M | CHEMI |
| 50,000/mL E.c. | NEG | GOLD |
| E. coli | E.c.-S, Kl.-M | CHEMI |
| E. coli | E.c.-VS, Kl.-S | CHEMI |
| E. coli | E.c.-S, Kl.-S | CHEMI |
| E. coli/Klebsiella mix | E.c.-S, Kl.-W | GOLD |
| E. coli/Staph mix | E.c.-S, St.-M | CHEMI |

Table 1 cont'd

| HOSPITAL DIAGNOSIS | APPLICANTS' HYBRIDIZATION RESULTS | DETECTION SYSTEM |
|---|---|---|
| Klebsiella spp. | E.c.-M, Kl.-W | CHEMI |
| 100,000/mL K. pneumoniae | E.c.-W, Kl-VW | GOLD |
| Enterobacter spp. | NEG** | GOLD |
| 100,000 Candida | NEG** | GOLD |
| 100,000/mL Proteus | Pr.-S, E.c.-W | GOLD |
| 10,000/mL Strep | NEG | CHEMI |
| Mixture of 3 unidentified Gm(+) | NEG | GOLD |

* diagnosis conducted by streaking urine on an agar plate and treating the plate under conditions so that the infectious organism can grow.

** Enterobacter/Candida probes not included in the hybridization assay, therefore, negative results are not surprising; given the high stringency conditions employed in the assay, cross-hybridization with species related to Enterobacter was not detected.

Abbreviations: VS=very strong; S=strong; M=medium; W=weak; and VW=very weak hybridization signals; GOLD=detection method according to Example 12; CHEMI=chemiluminescent detection according to Example 3(b)

Applicants' hybridization results represent the results of a subjective interpretation of the intensity of the hybridization signals obtained after detection. DNAs from the organisams listed in column two are the only ones for which any hybridization signal was obtained. The panel of DNAs used for hybridization included E. coli ("E.c."), Klebsiella pneumoniae ("Kl"), Proteus vulgaris ("Pr"), Pseudomonas aeruginosa, Staphylococcus epidermatis ("SE"), Streptococcus faecalisand Homo sapiens.

TABLE 2

COMPARISON OF AMES LEUKOSTIX ASSAY WITH APPLICANT'S ASSAY

| "LEUKOSTIX" RESULT | APPLICANTS' HYBRIDIZATION RESULT | DETECTION SYSTEM |
|---|---|---|
| 3+ | VS | GOLD |
| 3+ | S | CHEMI |
| 3+ | S | CHEMI |
| 3+ | M | CHEMI |
| 3+ | M | CHEMI |
| 3+ | S | GOLD |
| 3+ | S | GOLD |
| 3+ | VS | GOLD |
| 3+ | VS | GOLD |
| 3+ | VS | GOLD |
| 3+ | VS | GOLD |
| 2+ | S | CHEMI |
| 2+ | S | CHEMI |
| 2+ | S | CHEMI |
| 2+ | S | CHEMI |
| 2+ | S | GOLD |
| 2+ | S | GOLD |
| 2+ | S | GOLD |
| 2+ | S | GOLD |
| 2+ | S | GOLD |
| 1+ | S | GOLD |
| 1+ | VS | GOLD |
| 1+ | VW | GOLD |
| TRACE/1+ | M | CHEMI |
| TRACE | VS | CHEMI |
| TRACE | W | GOLD |
| TRACE | W | GOLD |
| TRACE | VS | GOLD |
| NEG | S | CHEMI |
| NEG | S | CHEMI |
| NEG | M | CHEMI |
| NEG | VW | GOLD |

Table 2 cont'd

| "LEUKOSTIX" RESULT | APPLICANTS' HYBRIDIZATION RESULT | DETECTION SYSTEM |
|---|---|---|
| NEG | VW | GOLD |
| NEG | NEG | GOLD |
| NEG | NEG | GOLD |
| NEG | W | GOLD |
| NEG | W | GOLD |
| NEG | W | GOLD |

The hybridization results summarized in column 2 of Table 2 represent subjective interpretations of the intensity of hybridization signal obtained when labeled urine samples described in Table 1 were hybridized with genomic human DNA.

The "LEUKOSTIX" assay is a colorimetric reagent strip assay. Color development on the reagent strip is compared to a chart provided with the assay reagent strips and ranges from negative (no color development) to 3+ (very strong color development).

Example 14: Lysis of Cells

A 1.0 mL aliquot of cell suspension was centrifuged and the cell pellet resuspended in 100 µL of unbuffered NaOH solution. The sample was then exposed to high temperature for a short time and then diluted to the original volume using 10 mM borate buffer. The pH of the solution was then adjusted to neutral with HCl.

Table 4 shows the efficiency of lysis of two different Gram positive cocci, Staphylococcus epidermidis and Streptococcus faecalis, at varying NaOH concentrations at either 68°C or 100°C. In this Example, the absorbance of the 10 mL aliquots at 600 nm was recorded before centrifugation. After centrifugation, the cell pellets were resuspended in varying concentrations of NaOH (100 µL) and duplicate samples of each exposed to 68°C for 10 minutes or 100°C for 5 minutes. Each sample was then diluted to 1.0 mL and the absorbance at 600 nm again recorded. Since the beginning and ending volumes are identical, the beginning and ending absorbance at 600 nm provides a direct measurement of lysis efficiency.

Whereas Gram negative organisms lysed efficiently in as low as 0.1 N NaOH, Table 4 shows clearly that efficient lysis is a function of both NaOH concentration and temperature, such that higher NaOH concentrations are required as the incubation temperature decreases. At 100°C (maximum temperature at 1 atmosphere) a concentration of at least 1.6 N NaOH was required for efficient lysis of S. epidermidis and S. faecalis. If lower temperatures are desirable or necessary, then higher concentrations of NaOH will be required to maintain lysis efficiency.

TABLE 3

EFFICIENCY OF LYSIS OF GRAM POSITIVE BACTERIA AT VARIOUS CONCENTRATIONS OF NaOH AT 68°C and 100°C

Streptococcus faecalis

| | 100°C/5 Minutes | | | 68°C/10 Minutes | | |
|---|---|---|---|---|---|---|
| [NaOH] | OD600 PRE | OD600 POST | %LYSIS | OD600 PRE | OD600 POST | %LYSIS |
| 0 N | 0.475 | 0.366 | 23 | 0.512 | 0.357 | 30 |
| 0.1 | .509 | .261 | 50 | .513 | .238 | 54 |
| 0.2 | .512 | .194 | 62 | .514 | .259 | 50 |
| 0.4 | .504 | .175 | 65 | .513 | .150 | 71 |
| 0.8 | .506 | .113 | 78 | .505 | .147 | 71 |
| 1.2 | .498 | .082 | 84 | .498 | .150 | 70 |
| 1.6 | .487 | .061 | 88 | .426 | .099 | 77 |

Staphylococcus epidermidis

| | 100°C/5 Minutes | | | 68°C/10 Minutes | | |
|---|---|---|---|---|---|---|
| [NaOH] | OD600 PRE | OD600 POST | %LYSIS | OD600 PRE | OD600 POST | %LYSIS |
| 0 N | 0.667 | 0.558 | 16 | 0.690 | 0.560 | 19 |
| 0.1 | .681 | .396 | 42 | .701 | .441 | 37 |
| 0.2 | .674 | .296 | 60 | .699 | .414 | 41 |
| 0.4 | .699 | .183 | 74 | .730 | .309 | 58 |
| 0.8 | .705 | .091 | 87 | .715 | .187 | 74 |
| 1.2 | .680 | .070 | 90 | .719 | .090 | 88 |
| 1.6 | .693 | .035 | 95 | .660 | .040 | 94 |

It will be appreciated that the instant specification and claims are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

## Claims

1. A method for detecting one or more microorganisms or polynucleotide sequences from eukaryotic sources in a nucleic acid-containing test sample comprising

a) preparing a test sample comprising labeling the nucleic acids in the test sample,

b) preparing one or more probes by immobilizing an oligonucleotide or a single-stranded nucleic acid of one or more known microorganisms or sequences from eucaryotic sources,

c) contacting, under hybridization conditions, the labeled single-stranded sample nucleic acid and the immobilized oligonucleotide or single-stranded nucleic acid to form hybridized labeled nucleic acids, and

d) assaying for the hybridized nucleic acids by detecting the label.

2. A method according to claim 1, further comprising denaturing the labeled nucleic acids to form labeled single stranded nucleic acids.

3. A method according to any of claims 1 and 2, wherein said eukaryotic sources are selected from the group consisting of algae, protozoa, fungi slime molds and mammalian genetic defects, such as alpha-thalassemia and sickle cell anemia.

4. A method according to any of claims 1 to 3, wherein the labeling is conducted in a whole living cell or a cell lysate.

5. A method according to claim 4, wherein the cell lysate is prepared by contacting a cell with alkali.

6. A method according to any of claims 1 to 5, wherein the label is selected from the group consisting of protein binding ligands, haptens, antigens, fluorescent compounds, dyes, radioactive isotopes and enzymes.

7. A method according to any of claims 1 to 6, wherein the immobilization is carried out by chemical reaction or physical adsorption.

8. A method according to any of claims 1 to 7, wherein the probe comprises the two or more known microorganisms or sequences from eukaryotic sources immobilized in the form of dots on a solid support strip.

9. A method according to any of claims 1 to 8, wherein said labeling is carried out by photochemically reacting a nucleic acid binding ligand with the nucleic acid in the test sample.

A kit for detecting one or more microorganisms or polynucleotide sequences from eukaryotic sources in a test sample comprising in one or more containers

a) a solid support containing single-stranded nucleic acids of one or more known microorganisms or polynucleotide sequences from eukaroytic sources immobilized thereon,

b) a reagent for labeling the nucleic acid of the test sample,

c) a reagent for denaturing nucleic acid in the test sample, and

d) hybridization reagents.

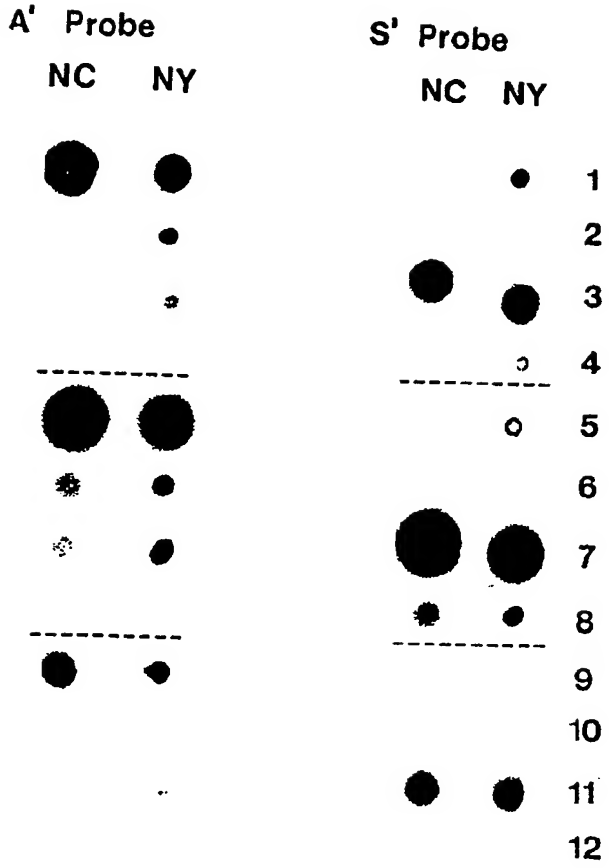



FIG. 1

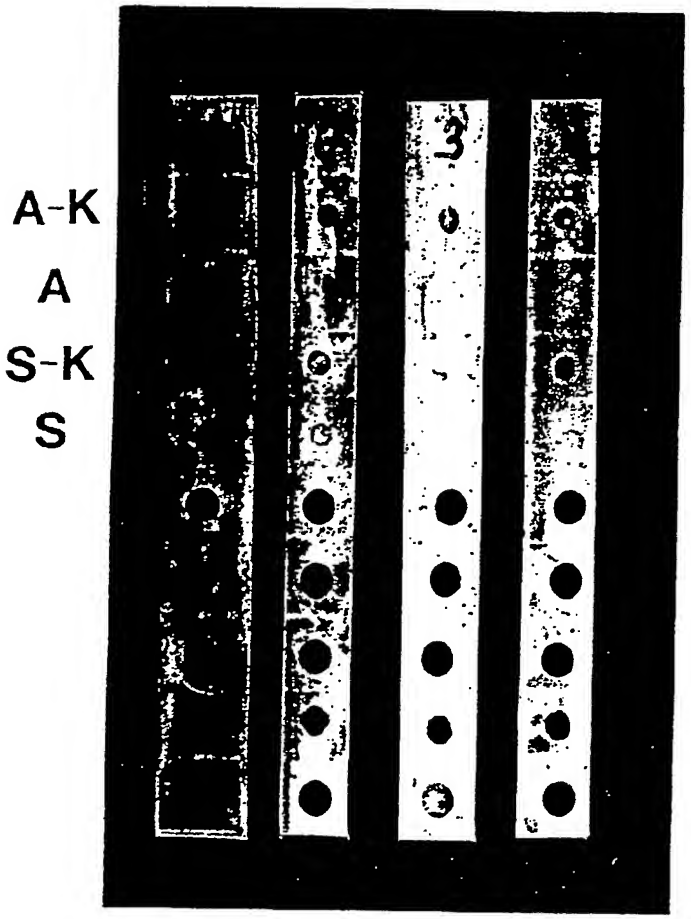



FIG. 2